



Protozoan and viral pathogens of grasshoppers
by John Edward Henry

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Entomology
Montana State University
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Abstract:

The development and pathologies of some protozoan and viral pathogens of grasshoppers are described and discussed in terms of the experimental and natural host-pathogen relationships. Included among the protozoans are: (1) *Malameba locustae*, an amoebic organism which infects tissues of the digestive tract and causes physiological changes which are reflected in the host's reproduction, (2) undetermined species of *Eugregarinida* which are common in grasshoppers but are considered relatively avirulent, (3) an undescribed species of *Neogregarinida* which from the observed stages of schizogony and sporogony probably belongs to the genus *Caulleryella*, (4) *Nosema locustae*, a species of *Microsporidia* that infects primarily the fat bodies of grasshoppers, (5) *Nosema acridophagus* which infects a number of host tissues in which it induces cellular aggregations described as tumors, (6) an undescribed species of *Nosema* which infects various host tissues including those of the nervous system, and (7) an undetermined number of undescribed organisms which, based on the relative size of the spore, are referred to as small spore organisms, but which exhibit developmental stages that are typical of species of *Microsporidia*. Included among the viruses are (1) the grasshopper inclusion body virus (GIBV) which is a DNA-containing virus that infects the fat bodies of the host and exhibits replicating forms resembling those reported during the development of pox viruses, and (2) the crystalline-array virus (CAV) which is a small RNA-containing virus that infects the pericardium, muscles and tracheal matrix of the host and is the most virulent of the reported grasshopper pathogens.

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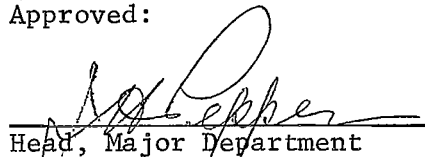
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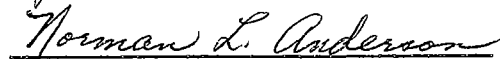
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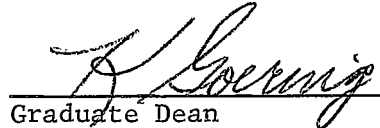
in

Entomology

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Bozeman, Montana

June, 1969

ACKNOWLEDGMENT

I wish to thank Dr. N. L. Anderson for his help and advice while serving as chairman of the graduate committee, and Dr. J. H. Pepper, Dr. P. D. Skaar, Dr. R. H. McBee, and Dr. J. W. Jutila for serving on the committee. Also, I thank Dr. Jutila for allowing the inclusion of results from cooperative studies in this thesis. I am grateful to F. T. Cowan, Investigations Leader (retired July 1, 1967), Grasshopper Investigations, for his supporting and encouraging these studies. I thank Dr. C. C. Blickenstaff, Investigations Leader, Grasshopper Investigations, for supporting these studies. I am grateful to Mrs. Barbara McCleave, Mrs. Elaine Oma, and Mrs. Barbara Nelson for their assistance during these studies, and to Mrs. Ruth Breeden for her assistance in the preparation of this manuscript. Also, I am grateful to Dr. Dave Worley for reviewing the manuscript.

TABLE OF CONTENTS

	Page
VITA.....	ii
ACKNOWLEDGMENT.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	xiv
INTRODUCTION.....	1
MATERIALS AND METHODS.....	3
Field Collection Techniques.....	3
Rearing Techniques.....	3
Preparation of Inoculants.....	4
Experimental Inoculation.....	5
Light Microscopic Techniques.....	5
Preparation of Smears.....	6
Preparation of Sectioned Tissues for Light Microscopy.....	7
Photomicroscopy.....	8
Preparation of Tissues for Electron Microscopy.....	9
RESULTS.....	10
<u>Malameba locustae</u> (King and Taylor) (Amoebida).....	10
Host and Geographical Range of <u>M. locustae</u>	10
Description of Stages in the Life Cycle of <u>M. locustae</u>	11
Histopathology Resulting from Infections by <u>M. locustae</u> ...	16
Transmission of <u>M. locustae</u>	19
Control of <u>M. locustae</u> in Grasshoppers in	
Laboratory Cultures.....	20
Eugregarinida.....	22
Differentiation of Eugregarinida from Grasshoppers.....	23
Host and Geographical Ranges of Eugregarinida in	
Grasshoppers.....	25
Generalized Life Cycle of Eugregarinida in Grasshoppers...	26
Stages in the Life Cycle of Eugregarinida in	
Grasshoppers.....	28
Histopathology of Infections by Eugregarinida in	
Grasshoppers.....	41
Source of Infections by Eugregarinida in Grasshoppers.....	42

	Page
Neogregarinida.....	43
Host and Geographical Range of Neogregarinida	
in Grasshoppers.....	43
Stages in the Life Cycle of the Neogregarinida	
in Grasshoppers.....	44
Proposed Life Cycle of the Neogregarinida	
from Grasshoppers.....	50
Histopathology of Infections by the Neogregarinida	
from Grasshoppers.....	52
Microsporida.....	53
Life Cycle of <u>Nosema</u> spp. in Grasshoppers.....	54
<u>Nosema locustae</u> Canning.....	56
Host and Geographical Range of <u>N. locustae</u>	56
Stages in the Life Cycle of <u>N. locustae</u>	60
Histopathology of Infections by <u>N. locustae</u>	63
Effect of Infections by <u>N. locustae</u> on Grasshoppers.....	64
Transmission of <u>N. locustae</u>	71
<u>Nosema acridophagus</u> Henry.....	71
Host and Geographical Range of <u>N. acridophagus</u>	71
Stages in the Life Cycle of <u>N. acridophagus</u>	72
Histopathology of Infections by <u>N. acridophagus</u>	75
Gross Effects of Infections by <u>N. acridophagus</u>	82
Transmission of <u>N. acridophagus</u>	85
A <u>Nosema</u> sp.....	85
Host and Geographical Range of the <u>Nosema</u> sp.....	85
Stages in the Life Cycle of the <u>Nosema</u> sp.....	85
Histopathology of Infections by the <u>Nosema</u> sp.....	87
Gross Effects of Infections by the <u>Nosema</u> sp.....	90
Transmission of the <u>Nosema</u> sp.....	92
Comparative Susceptibility of <u>Melanoplus bivittatus</u> to	
<u>Nosema locustae</u> , <u>Nosema acridophagus</u> , and the <u>Nosema</u> sp...	92
Small Spore Diseases of Grasshoppers.....	96
The Grasshopper Inclusion Body Virus (GIBV).....	100
Host and Geographical Range of the GIBV.....	100
Morphological and Chemical Characterization of the GIBV...	100
Histopathology of Infections by the GIBV.....	107
Replication of the GIBV.....	108
Gross Effects of Infection by the GIBV.....	114
Observations of the Natural Occurrence of the GIBV.....	115
Effect of Host Nutrition on the Development of the GIBV...	116
Relationship Between the GIBV and Other Insect Viruses....	119
The Crystalline-array Virus.....	120
Host and Geographical Range of the CAV.....	121
Morphological and Chemical Characterization of the CAV....	121
Replication of the CAV.....	127
Gross Effects of Infections by the CAV on Grasshoppers....	130
Similarities Between the CAV and Other Viruses.....	132

	Page
DISCUSSION AND CONCLUSIONS.....	133
SUMMARY.....	144
FOOTNOTES.....	147
LITERATURE CITED.....	148

LIST OF TABLES

Table		Page
I	Average dimensions of cysts of <u>Malameba locustae</u> from <u>Melanoplus bivittatus</u> , <u>Melanoplus differentialis</u> , and <u>Melanoplus sanguinipes</u>	15
II	Species of Orthoptera that are susceptible to infections by <u>Nosema locustae</u> and the sources of the observed infections.....	58
III	Susceptibility of <u>Melanoplus bivittatus</u> to infections by <u>Nosema locustae</u>	65
IV	Effect of infections by <u>Nosema locustae</u> on the rate of development of <u>Melanoplus sanguinipes</u> as determined by the frequency of adults in the test groups.....	69
V	Effect of infections by <u>Nosema locustae</u> Canning on the fecundity of <u>Melanoplus differentialis</u>	70
VI	Susceptibility of <u>Melanoplus bivittatus</u> to infections by <u>Nosema acridophagus</u>	84
VII	Susceptibility of <u>Melanoplus bivittatus</u> to infections by the <u>Nosema</u> sp.....	91
VIII	Effect of different host diets on infection by the grasshopper inclusion body virus in <u>Melanoplus sanguinipes</u>	118
IX	Cumulative percent mortality among third instar nymphs of <u>Melanoplus bivittatus</u> and <u>M. sanguinipes</u> after <u>per os</u> inoculation with CAV.....	131

LIST OF FIGURES

	Page
Fig. 1. Fresh preparation under phase contrast microscopy of cysts and trophozoites of <u>Malameba locustae</u>	14
Fig. 2. Cross section of Malpighian tubules of <u>Melanoplus sanguinipes</u> with trophozoites of <u>Malameba locustae</u> on the inner surface of the walls and cysts in the lumen of the tubules.....	14
Fig. 3. Cross section of the posterior region of the midgut of a <u>Melanoplus sanguinipes</u> showing the deterioration of part of the epithelium due to infection by <u>Malameba locustae</u>	14
Fig. 4. Fresh preparation under phase contrast microscopy of a Malpighian tubule of a <u>Melanoplus sanguinipes</u> heavily compacted with cysts of <u>Malameba locustae</u>	14
Fig. 5. Fixed and stained preparation from a <u>Melanoplus sanguinipes</u> showing the cystocytic encapsulation of a Malpighian tubule containing cysts of <u>Malameba locustae</u>	14
Fig. 6. Distributions of measurements of cysts of Eugregarinida from <u>Melanoplus bruneri</u> , <u>Melanoplus oregonensis</u> , <u>Bruneria brunnea</u> , <u>Camnula pellucida</u> , <u>Chorthippus curtipennis</u> , and <u>Neopodismopsis abdominalis</u> and <u>Melanoplus bruneri</u> alone which were collected in the Antelope Basin region of southwestern Montana.....	24
Fig. 7. Diagrammatic representation of the life cycle of Eugregarinida as determined from stages observed during studies of infections in grasshoppers.....	27
Fig. 8. Mature spores extruded from a cyst of Eugregarinida from a grasshopper.....	30
Fig. 9. Sporoblasts from a cyst of Eugregarinida from a grasshopper.....	30
Fig. 10. Fresh preparation under phase contrast microscopy of a sporozoite which was extruded from a spore of Eugregarinida from a grasshopper.....	30
Fig. 11. Young cephalont of a Eugregarinida with the epimerite segment embedded in the cytoplasm of an epithelial cell of a gastric caecum of <u>Melanoplus sanguinipes</u>	30

Fig. 12.	Fresh preparation under phase contrast microscopy showing a group of cephalonts of Eugregarinida in tissues of a gastric caecum of a grasshopper.....	30
Fig. 13.	Various sized sporonts around the satellite of an association of Eugregarinida from the lumen of the midgut of a grasshopper.....	34
Fig. 14.	An association consisting of an anterior sporont (primita) and a posterior sporont (satellite).....	34
Fig. 15.	Fresh preparation under phase contrast microscopy of the nucleus of a sporont of Eugregarinida showing the beaded arrangement of the karyosomes around a nucleolus.....	34
Fig. 16.	Sporonts and newly formed gametocysts of Eugregarinida from a <u>Melanoplus sanguinipes</u>	34
Fig. 17.	Newly formed sporoblasts in a sectioned preparation of a cyst of Eugregarinida from grasshoppers.....	39
Fig. 18.	Cross section of a gastric caecum from a <u>Melanoplus bruneri</u> showing cephalonts of Eugregarinida attached to the epithelium.....	39
Fig. 19.	Cross section of the anterior part of the midgut of a <u>Melanoplus bruneri</u> in which the lumen is nearly filled with trophozoites of Eugregarinida.....	39
Fig. 20.	Fecal pellets from a <u>Melanoplus sanguinipes</u> with attached cysts of Eugregarinida.....	39
Fig. 21.	Fresh preparation under phase contrast microscopy showing uninucleate, binucleate and trinucleate schizonts of the Neogregarinida of grasshoppers.....	46
Fig. 22.	A terminal multinucleate schizont of the Neogregarinida of grasshoppers.....	46
Fig. 23.	A vermiform trinucleate schizont of the Neogregarinida of grasshoppers which was observed in fresh preparations of tissues from the hemocoel of a grasshopper.....	46
Fig. 24.	A granular multinuclear schizont of the Neogregarinida of grasshoppers observed in a smear preparation of the hemolymph of a grasshopper.....	46

- Fig. 25. A group of granular gametocytes of the Neogregarinida observed in a fresh preparation of the hemolymph of a grasshopper..... 49
- Fig. 26. Sectioned preparation of the fat body and pericardial tissues of a grasshopper showing a heavy concentration of gametocytes, some of which are conjugated, and host pericardial cells which show indications of deterioration..... 49
- Fig. 27. Cysts of the Neogregarinida of grasshoppers which exhibit different stages of development..... 49
- Fig. 28. Cysts of the Neogregarinida of grasshoppers which contain mature appearing spores..... 49
- Fig. 29. Diagrammatic representation of the proposed life cycle of the Neogregarinida from grasshoppers..... 51
- Fig. 30. Diagrammatic representation of the generalized life cycle of Nosema spp. as determined from the stages observed during studies of the life cycle of Nosema acridophagus..... 55
- Fig. 31. Binucleate sporonts of Nosema locustae in a smear preparation..... 62
- Fig. 32. Spores and sporoblasts of Nosema locustae in a smear preparation..... 62
- Fig. 33. Spores, sporoblasts, and a megaspore of Nosema locustae under phase contrast microscopy..... 62
- Fig. 34. Spore of Nosema locustae as observed during extrusion of the polar filament..... 62
- Fig. 35. Section of the fat bodies and nerve cords of a nymph of Melanoplus sanguinipes showing the heavy accumulations of spores of Nosema locustae..... 62
- Fig. 36. The rate of mortality among individually reared Melanoplus sanguinipes which were inoculated with 4×10^6 and 4×10^4 spores of Nosema locustae..... 67
- Fig. 37. A binucleate schizont of Nosema acridophagus..... 74

	Page
Fig. 38. A quadrinucleate schizont of <u>Nosema acridophagus</u>	74
Fig. 39. A diplokaryon form of <u>Nosema acridophagus</u>	74
Fig. 40. A binucleate sporont of <u>Nosema acridophagus</u>	74
Fig. 41. A spindle-shaped sporoblast of <u>Nosema acridophagus</u>	74
Fig. 42. A spore-shaped sporoblast of <u>Nosema acridophagus</u>	74
Fig. 43. A spore of <u>Nosema acridophagus</u> with an extruded polar filament.....	74
Fig. 44. Schizonts and sporonts of <u>Nosema acridophagus</u> in a binucleate pericardial cell.....	77
Fig. 45. Cross section of the convoluted epithelium of a gastric caecum from <u>Melanoplus sanguinipes</u> showing initial tumor formation, as indicated by the enlarged regenerative nidi, during infection by <u>Nosema acridophagus</u>	77
Fig. 46. Cross section of the midgut of a <u>Melanoplus sanguinipes</u> showing the appearance of a tumor during initial formation and some vacuolization of the epithelial cells...	77
Fig. 47. Cross section of a gastric caecum of a <u>Melanoplus</u> <u>sanguinipes</u> which was prepared at 48 hours after inoculation with <u>Nosema acridophagus</u> showing a tumor which encompasses schizonts of the microsporidian.....	81
Fig. 48. Cross section of the midgut of a <u>Melanoplus sanguinipes</u> showing a tumor and spores and sporonts of <u>Nosema</u> <u>acridophagus</u> between layers of the basal membrane.....	81
Fig. 49. Cross section of a gastric caecum of a <u>Melanoplus</u> <u>sanguinipes</u> prepared at 72 hours after inoculation with <u>Nosema acridophagus</u> showing a large tumor with a melanotic core.....	81
Fig. 50. Cross section of the posterior region of the midgut of a <u>Schistocerca americana</u> showing tumors induced by <u>Nosema acridophagus</u> encircling the digestive tract.....	81
Fig. 51. Fresh preparation under phase contrast microscopy of the spores of <u>Nosema</u> sp. showing an extruded polar filament from a common-sized spore.....	89

	Page
Fig. 52. Fresh preparation under phase contrast microscopy showing spores of <u>Nosema</u> sp. and an extruded polar filament from a megaspore.....	89
Fig. 53. Rates of mortality among individually-reared <u>Melanoplus bivittatus</u> following inoculation with <u>Nosema locustae</u> , <u>Nosema acridophagus</u> , or <u>Nosema</u> sp.....	94
Fig. 54. Rates of mortality among group-reared <u>Melanoplus bivittatus</u> following inoculation with <u>Nosema locustae</u> , <u>Nosema acridophagus</u> , or <u>Nosema</u> sp.....	95
Fig. 55. Section of the fat bodies of a <u>Hadrotettix trifasciatus</u> showing spores, sporonts and schizonts of a small spore type organism.....	99
Fig. 56. Inclusion of the GIBV in a fresh preparation under bright light microscopy.....	102
Fig. 57. Inclusions of the GIBV in a sectioned preparation of the fat bodies of a <u>Melanoplus sanguinipes</u>	102
Fig. 58. Fresh preparation under phase microscopy of inclusions of the GIBV during initial degradation in a 0.1% solution of sodium hydroxide.....	102
Fig. 59. Fresh preparation under phase microscopy of the expanded inclusions of the GIBV containing the viral particles following treatment with a 0.1% solution of sodium hydroxide.....	102
Fig. 60. Ultra-thin section of an inclusion of the GIBV during development showing the regular dispersal of viral particles.....	105
Fig. 61. Ultra-thin section of a developing inclusion of the GIBV showing the structure of the viral particles.....	105
Fig. 62. Ultra-thin section of a fat body cell of <u>Melanoplus sanguinipes</u> showing fibrillar-granular and granular precursor masses, and presumptive viral particles "budding" off the granular masses as observed during infections by the GIBV.....	110

	Page
Fig. 63. Ultra-thin section of a fat body cell from <u>Melanoplus sanguinipes</u> showing granular precursor masses and "budding" presumptive viral particles.....	110
Fig. 64. Ultra-thin section of a fat body cell of <u>Melanoplus sanguinipes</u> showing viral particles and an initial inclusion of the GIBV.....	113
Fig. 65. Fresh preparation of the tracheal matrix of a <u>Schistocerca americana</u> containing crystals of the CAV.....	124
Fig. 66. Ultra-thin section under light microscopy showing crystals of the CAV in the muscles of <u>Schistocerca americana</u>	124
Fig. 67. Ultra-thin section of a pericardial cell of <u>Schistocerca americana</u> showing the crystalline array arrangement of viral particles in a developing crystal...	126
Fig. 68. Ultra-thin section of a pericardial cell showing developing crystals of the CAV in an electron dense replicating matrix.....	126
Fig. 69. Ultra-thin cross section of the muscles of <u>Schistocerca americana</u> containing several vesicular membrane-like structures encircling viral particles.....	129

ABSTRACT

The development and pathologies of some protozoan and viral pathogens of grasshoppers are described and discussed in terms of the experimental and natural host-pathogen relationships. Included among the protozoans are: (1) Malameba locustae, an amoebic organism which infects tissues of the digestive tract and causes physiological changes which are reflected in the host's reproduction, (2) undetermined species of Eugregarinida which are common in grasshoppers but are considered relatively avirulent, (3) an undescribed species of Neogregarinida which from the observed stages of schizogony and sporogony probably belongs to the genus Caulleryella, (4) Nosema locustae, a species of Microsporidia that infects primarily the fat bodies of grasshoppers, (5) Nosema acridophagus which infects a number of host tissues in which it induces cellular aggregations described as tumors, (6) an undescribed species of Nosema which infects various host tissues including those of the nervous system, and (7) an undetermined number of undescribed organisms which, based on the relative size of the spore, are referred to as small spore organisms, but which exhibit developmental stages that are typical of species of Microsporidia. Included among the viruses are (1) the grasshopper inclusion body virus (GIBV) which is a DNA-containing virus that infects the fat bodies of the host and exhibits replicating forms resembling those reported during the development of pox viruses, and (2) the crystalline-array virus (CAV) which is a small RNA-containing virus that infects the pericardium, muscles and tracheal matrix of the host and is the most virulent of the reported grasshopper pathogens.

INTRODUCTION

Studies of the pathogens of insects are justified because (1) they exist; (2) they function in the dynamics of insect populations; (3) they are potential problems in the maintenance of beneficial insects in nature and the laboratory; and (4) they are potentially useful in the control of pest insects. Steinhaus (1963) defines insect pathology as a "study of whatever 'goes wrong' with an insect", and includes both infectious and noninfectious diseases, with the latter including such manifestations as genetic aberrations, nutritional deficiencies, physical and chemical injuries, etc. However, such a definition becomes unwieldy because it infers that insect pathology includes anything which is abnormal and since it is often impossible to define what is normal it is equally impossible to define what is abnormal. For this reason most studies of the pathologies of insects, including these studies of pathogens of grasshoppers, have involved descriptions of the disease manifestations resulting in the presence of some observable microorganism. Generally such studies have been directed at characterizing microorganisms, describing their effect on the host, and evaluating their potential use as agents for the microbial control of pest insects, which in this case has been species of grasshoppers.

Prior to these studies the only protozoans isolated from grasshoppers were a species of Amoebida, Malameba locustae (King and Taylor), a species of Microsporida, Nosema locustae Ganning, and from about 25 to 40 species of Eugregarinida. While these studies were in progress, Steinhaus and Marsh (1962) and Bucher (1966) reported the isolation of an undetermined species of Neogregarinida. M. locustae, N. locustae, the undescribed

neogregarine, and several species of Eugregarinida were isolated and studied during these investigations. In addition, two previously undescribed species of microsporidia were isolated. One species, Nosema acridophagus Henry, was described (Henry, 1967) while the other species has been characterized sufficiently to place it in the genus Nosema Nageli. Also, several other probable microsporidians, which have been labeled as small spore diseases for purposes of reference, were observed in grasshoppers. However, because these organisms were not experimentally transmitted, their generic characteristics were not established.

In addition to the studies of Protozoa, these investigations resulted in the isolation of the only viruses known from grasshoppers. The preliminary characterization of the grasshopper inclusion body virus (GIBV) was reported by Henry and Jutila (1966). Subsequent reports of these viruses will be published jointly with Dr. J. W. Jutila^{1/}

MATERIALS AND METHODS

The following techniques were routinely used throughout these studies. Specialized techniques will be described in the sections dealing with the organisms with which they were employed.

Field Collection Techniques

Grasshoppers usually were collected in the field with sweep nets. The collected grasshoppers were either reared individually in glass or plastic tubes, or frozen at the time of collection. The reared grasshoppers were either killed and examined after a period of post collection rearing or examined when they died. The frozen grasshoppers were examined according to the routine procedures described below.

In 1963 a study was initiated on the natural occurrence of Nosema locustae in grasshoppers in Camas County, Idaho. Fifty-two permanent sampling sites were established within the 200 square mile study area. During 1963 to 1968 each site was sampled periodically during the summer seasons. Sampling consisted of recording the time of collection, atmospheric temperatures (at about 3 feet above the surface of the ground), wind direction and speed, and the grasshopper density as determined from 40 sweeps along predetermined transects. Also up to 30 grasshoppers were collected and frozen at each sampling period from each site. In the laboratory the frozen grasshoppers were separated by species, sex and stage of development. They were then examined for the presence or absence of N. locustae or other pathogens.

Rearing Techniques

The following species of grasshoppers were used for most laboratory studies: Melanoplus bivittatus (Say), Melanoplus sanguinipes (Fabricius),

Melanoplus differentialis (Thomas), Schistocerca vaga vaga (Scudder), and Schistocerca americana (Drury). The three species of Melanoplus were obtained from eggs collected in the field or from eggs oviposited in the laboratory by field-collected grasshoppers. S. vaga vaga and S. americana were reared in the laboratory and the breeding cultures were held in metal cages (Mazuranich and Cowan, 1966) in a greenhouse under a 16-hour daylight regime.

The eggs of S. vaga vaga and S. americana were incubated in moistened sand at 30° C. and usually hatched 19 to 25 days after deposition. The eggs of the Melanoplus species were incubated for 2 weeks at room temperature, then 3 months at 4° C. and then until they hatched, usually about 10 days, at 30° C. The nymphs were reared in an incubator room or in growth chambers at 30° C. with continuous illumination. They were fed daily with fresh lettuce, head or Romaine, and wheat bran. In most cases the diet was supplemented with an artificial medium similar to that reported by Kreasky (1962) but which also contained wheat germ, dried lettuce powder, and Cerophyl.^{2/}

Preparation of Inoculants

Inoculants were prepared by homogenizing infected grasshoppers in distilled water using 30-ml tissue grinding vessels with teflon pestles. Inoculants for injection were prepared further by passage through double thickness cheesecloth, followed by low speed centrifugation (3700 to 4500 rpm) and then passing the supernatant through a series of millipore filters down to filters with average pore sizes of 0.80 micron or less.

Experimental Inoculations

The inoculants were administered either per os or by intrahemocoelic injection. Per os applications were made either by placing a drop of the inoculant onto the mouthparts of the grasshopper or by placing a drop onto a lettuce disc (about 5 mm in diameter) which was then fed to a grasshopper that had been starved for about 24 hours prior to treatment. Injections were made intrathoracically using a 27-gauge needle mounted on a $\frac{1}{4}$ cc syringe. Precise volumes of pathogenic organisms were administered by mounting the syringe on a specially adapted micrometer caliper which, with a $\frac{1}{4}$ cc syringe, delivered inoculants within 4% of the desired volumes. The volume was preadjusted so as to yield the desired concentration in a 5 microliter drop. In tests involving small nymphs the volumes were adjusted to deliver 2.5 μ l per drop. The concentrations of pathogens in the inoculating media were determined by direct counts using a hemacytometer.

Light Microscopic Techniques

Preliminary disease diagnosis was accomplished by examining specific organs or tissues of dissected grasshoppers for abnormalities using a dissecting microscope. Infections by gregarines were diagnosed by the detection of cysts on fecal pellets using a dissecting microscope. Preliminary characterization of the histopathology and etiological agents was based on the abnormal appearance of tissues under phase contrast microscopy. All the pathogens reported here were recognized under phase contrast microscopy at magnifications of 500 to 1250 diameters.

Routine examinations to determine only the presence or absence of

particular pathogens were conducted by homogenizing grasshoppers and examining the homogenates as hanging drops under bright light microscopy. For more critical examinations and for detection of infection by the crystalline-array virus (CAV), the homogenates were examined under a cover glass using phase contrast microscopy.

Estimates of the levels of infection (number of pathogens per grasshopper weight) for all pathogens except the CAV were conducted by weighing each grasshopper and homogenizing each in a measured amount of water. The concentrations of pathogens in the homogenates were determined by direct counts using a hemacytometer. For comparative purposes the counts were expressed as the number of organisms per milligram (mg) of grasshopper weight. Estimates of the level of infection by M. locustae also were made by counting the number of cysts per mg of feces.

Preparation of Smears

Smears of homogenates or infected tissues were prepared to aid in the characterization of the etiological agents and for detection of various developmental stages. They were fixed in either methanol, osmic tetroxide fumes (2% solution), Bouin's solution, Gilson's fluid, Schaudinn's solution or by air drying. They were stained by the Giemsa technique or with various hematoxylin solutions. The most suitable technique for most protozoans in grasshoppers was fixation of dried smears in methanol for 2 minutes and, after drying, staining according to the Giemsa technique outlined by Galligher and Kozloff (1964). Depending on the susceptibility of the specimens to this stain, the smears were left in the staining solution (4% Giemsa stock solution in buffered water) for 15 minutes to

2 hours. The Giemsa stock solution consisted of 1 gm of Giemsa stain, 66 mls of glycerol and 66 mls of methanol. For more rapid staining, but with less control over the intensity of staining, smears were flooded with the stock solution for 2 to 5 minutes and differentiated for 2 to 5 minutes by adding an equal amount of buffered water. After staining the smears were washed for 5 minutes in tap water, air dried and mounted with Histoclad.^{3/} The smears were examined using bright light microscopy at magnifications up to about 1250 diameters.

Preparation of Sectioned Tissues for Light Microscopy

Tissue sections were prepared to aid in the characterization of etiological agents and histopathology. Although a number of the procedures listed by Galligher and Kozloff (1964) were used for preparation of sectioned tissues, the following procedure routinely produced the most satisfactory results with soft tissues from grasshoppers.

The tissues were fixed in Gilson's fluid for 24 hours or more, after which they were washed in 70% ethanol for at least 24 hours. They were dehydrated in 95% ethanol for 8 to 24 hours, then in absolute ethanol for 12 to 24 hours. They were then cleared in 1 to 3 parts xylene and absolute ethanol for 3 hours, followed by equal parts of xylene and absolute ethanol for 3 hours, followed by 3 to 1 parts xylene and absolute ethanol for 3 hours and then in xylene for 6 hours. The tissues were infiltrated in 3 to 1 parts xylene and melted Paraplast^{4/} for 3 to 6 hours, followed by equal parts of xylene and Paraplast for 3 to 6 hours, followed by 1 to 3 parts xylene and Paraplast for 3 to 6 hours, and then in several changes of Paraplast for 4 to 6 hours. Infiltration was conducted in an oven at

57° C. The tissues were embedded in Paraplast and cut in sections from 5 to 12 microns thick. The sections were affixed to slides using Mayers albumen or Haptas affixative and dried on a slide warmer for 4 to 12 hours. The sections were deparaffined according to the following sequence: 15 minutes in 2 changes of xylene; 2 minutes in equal parts of xylene and absolute ethanol; 2 minutes in absolute ethanol, 1 minute in 95% ethanol; 2 minutes in 70% ethanol; 1 minute in 40% ethanol, 1 minute in 15% ethanol; 2 to 3 minutes in distilled water. The sections were stained for 15 minutes in alum hematoxylin and washed in running tap water for 30 minutes. They were destained for 1 minute in each of 15%, 40%, and 70% ethanol. They were counterstained for 5 to 30 seconds in eosin, blotted, and destained for 2 minutes in 95% ethanol, 2 minutes in absolute ethanol and 5 minutes in xylene. They were mounted in Histoclad and examined at magnifications up to 1250 diameters.

Sections of tissues containing the grasshopper inclusion body virus were cut 5 microns thick and stained according to the procedures outlined by Hamm (1966). Thin sections, 0.2 to 0.5 micron thick, were prepared according to the procedures used by Harry (1965). The thin sections were prepared for studies of the small spore disease in Hadrotettix trifasciatus (Say) and some studies of gregarines.

Photomicroscopy

All photographs presented here of specimens under light microscopy were taken using a standard GFL Zeiss phase contrast microscope which was equipped with a basic body camera attachment and a Ukatron^{5/} microflash attachment. The photographic film was panatomic X (Kodak) with an ASA

rating of 32. The negatives were developed using a double bath developer (Diafine)^{6/} and Kodak fixer. The photographs were printed on hard gloss paper (Kodak medalist; F-3, F-4, or F-5).

Preparation of Tissues for Electron Microscopy

Most electron micrographs presented here were prepared according to the following procedure. Tissues were fixed in a 2.5% gluteraldehyde-potassium phosphate buffer (pH 7.0) for 15 minutes at room temperature, then postfixed in osmic tetroxide (2%) for 2 hours in a refrigerator maintained at about 4° C. They were dehydrated through a graded series of acetone followed by a final soaking in propylene oxide, after which they were embedded in Epon 6005^{7/}. After sectioning on a Reichart OM U2 Ultramicrotome, the sections were stained with aqueous uranyl acetate (2%) for 2 hours followed by Reynold's lead citrate for 5 minutes. They were examined with a Zeiss EM9 microscope. The electron micrographs were printed on hard gloss, high contrast paper (Kodabromide-5).

RESULTS

Malameba locustae (King and Taylor) (Amoebida)

Because of frequent chronic infections among laboratory-reared grasshoppers, most studies dealing with M. locustae involved the histopathology resulting from infection and the antibiotic control of the organism.

Host and Geographical Range of M. locustae

King and Taylor (1936) and Taylor and King (1937) described Malameba locustae from Melanoplus differentialis, M. sanguinipes (= M. mexicanus) and M. femurrubrum femurrubrum (DeGeer) in laboratory cultures in Iowa. In unpublished reports, G. T. York (in 1951) and R. L. Newton (in 1955) reported it from laboratory-reared grasshoppers at the Grasshopper Investigations Laboratory, USDA, at Bozeman, Montana. Steinhaus and Marsh (1962) reported M. locustae from Melanoplus bivittatus, M. sanguinipes (= M. mexicanus) and Melanoplus dawsoni (Scudder) which were sent to them by J. R. Parker of the Grasshopper Investigations Laboratory at Bozeman. In the Union of South Africa, Lea (1958) and Venter (1966) reported M. locustae in nature from brown locusts, Locustana pardalina (Walker), while Prinsloo (1960) studied it in L. pardalina in laboratory cultures. Recently Henry (1968) reported that infections were common in laboratory-reared grasshoppers at the Grasshopper Investigations Laboratory (USDA), Bozeman, Montana, and that he had observed it in grasshoppers and in grasshopper feces received from several other laboratories in the United States. Dr. Peggy Ellis (personal communication, 1968) of the Anti-Locust Research Centre, London, England, reported that M. locustae was common in laboratory cultures of various species of Schistocerca, Locusta, Nomadacris, and Chortoicetes.

Taylor and King (1937) listed 37 species and subspecies of grass-hoppers that were susceptible to experimental infections by M. locustae. These included 5 species in the subfamily Acridinae, 14 species or subspecies of Oedipodinae, and 18 species of Cyrtacanthacrinae. They also listed 12 species with which the results were inconclusive because of difficulty in maintaining these species in the laboratory.

During the present studies M. locustae has been diagnosed in Aeropedellus clavatus (Thomas), Melanoplus bruneri Scudder, Trachyrhachys kiowa kiowa (Thomas), Chortophaga viridifasciatus (DeGeer), and Psoloessa delicatula delicatula (Scudder) which were collected in Montana; M. sanguinipes, Melanoplus cuneatus Scudder, Aulocara ellioti (Thomas), Psoloessa delicatula, Eritettix variabilis Bruner, Hadrotettix trifasciatus (Say) and Xanthippus corallipes (Haldeman) from Arizona; and M. bivittatus from Idaho. In addition, trophozoites and cysts of M. locustae were observed in Schistocerca vaga vaga about 7 days after being placed in laboratory culture following their collection in southern California. Also in the laboratory, M. locustae has been found in Schistocerca americana, Melanoplus bivittatus, M. sanguinipes, M. differentialis, M. bruneri, Melanoplus oregonensis (Thomas), Melanoplus packardi Scudder, M. dawsoni, M. femurrubrum, Aulocara ellioti, Gamnula pellucida (Scudder), Hadrotettix trifasciatus, and Chorthippus curtipennis (Harris).

Description of the Stages in the Life Cycle of M. locustae

Under phase contrast microscopy, the trophozoites of M. locustae usually appeared spherical, somewhat transparent and measured from 4 to 12 microns in diameter (Fig. 1). Characteristically, the trophozoites

contained variable numbers of small, light-refractive granules.

Irregularly-shaped trophozoites were common which, as proposed by King and Taylor (1936), indicated that trophozoites moved by means of hemispherical or filiose pseudopodia. They also reported that reproduction occurred by binary fission during which each uninucleate trophozoite separated into two daughter cells. Multinucleate forms or stages representing sexual recombination have not been observed.

The cysts of M. locustae appeared ellipsoidal, measured about 12.5 microns in length by 7.8 microns in diameter in fresh preparations, possessed thick walls, and appeared refractive to light using phase contrast microscopy (Fig. 1). Most cysts contained a vacuole at one end and a variable number of light-refractive granules which appeared similar to those observed in the trophozoites. By subjecting cysts to the Feulgen reaction (Gallagher and Kozloff, 1964) it was determined that the nucleus existed as an elongate band which was located along one side of the cyst. The extrusion of young trophozoites from cysts was not observed.

King and Taylor (1936) reported that cysts measured about 9.6 microns long by 5.5 microns wide, but Prinsloo (1961) reported average measurements of 12 microns long by 8 microns in diameter. These differences probably resulted from their use of different techniques and different host species. Henry (1968) observed variations in measurements of cysts from three host species. As shown in Table I, the cysts measurements were consistently larger when the cysts were measured in fresh preparations than when measured in fixed and stained preparations. In fresh preparations, the cysts from Melanoplus differentialis measured less than those from

- Fig. 1. Fresh preparation under phase contrast microscopy of cysts (C) and trophozoites (T) of Malameba locustae. 750X.
- Fig. 2. Cross section of Malpighian tubules of Melanoplus sanguinipes with trophozoites of Malameba locustae on the inner surface of the walls and cysts in the lumen of the tubules. 450X.
- Fig. 3. Cross section of the posterior region of the midgut of a Melanoplus sanguinipes showing the deterioration of part of the epithelium due to infection by Malameba locustae. 64X.
- Fig. 4. Fresh preparation under phase contrast microscopy of a Malpighian tubule of a Melanoplus sanguinipes heavily compacted with cysts of Malameba locustae. 250X.
- Fig. 5. Fixed and stained preparation from a Melanoplus sanguinipes showing the cystocytic encapsulation of a Malpighian tubule containing cysts of Malameba locustae. 330X.

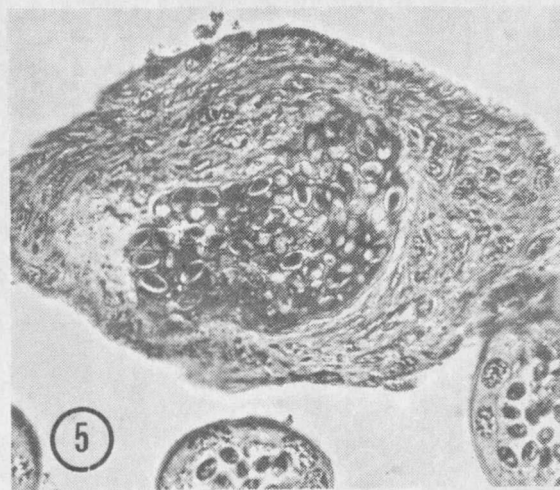
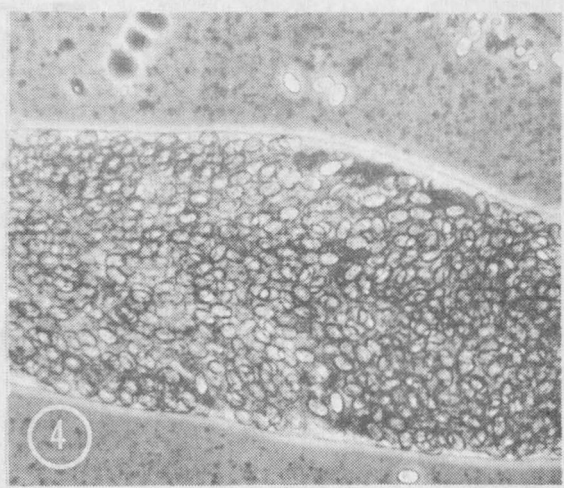
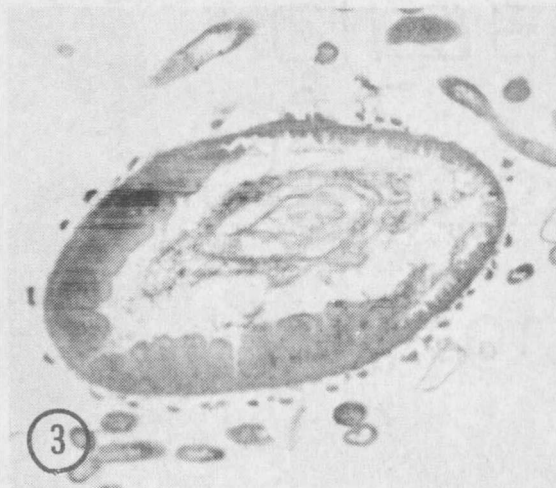
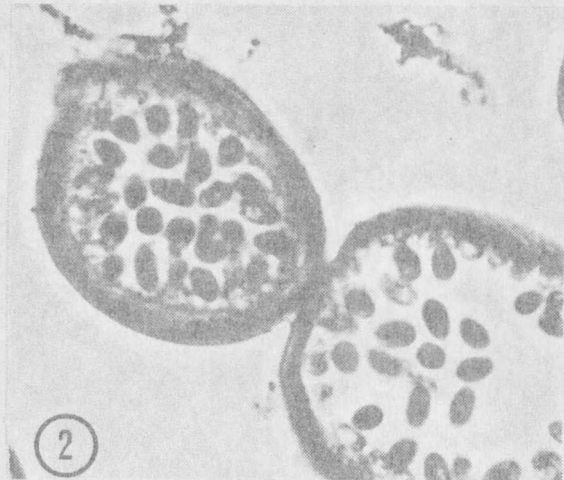
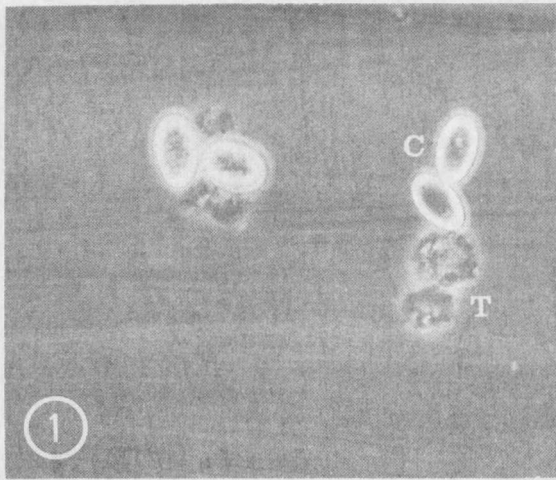


TABLE I

Average dimensions of cysts of Malameba locustae from Melanoplus bivittatus, Melanoplus differentialis, and Melanoplus sanguinipes. (Henry, 1968).

Host species ^a	Size in fresh preparation (μ)		Size in stained preparation (μ)	
	Length	Width	Length	Width
<u>M. bivittatus</u>	12.58	7.72	11.52	6.65
<u>M. differentialis</u>	12.25	7.58	11.27	7.22
<u>M. sanguinipes</u>	12.63	7.92	10.62	6.14

^a 50 cysts measured in each preparation.

either M. bivittatus or M. sanguinipes, but in fixed preparations the smallest cysts were those from M. sanguinipes.

As shown in Table I, the cysts from M. sanguinipes were larger than those from either M. bivittatus or M. differentialis when measured in fresh preparations, but smaller when measured in fixed preparations. During dissection it was noted that some of the Malpighian tubules of the M. sanguinipes were encapsulated and melanized. This condition was observed frequently in heavily infected individuals of various Melanoplus species. This host reaction possibly altered or weakened the cyst walls such that the cysts expanded more than normal in water and shrank more than normal when dehydrated in methanol.

Histopathology Resulting from Infections by *M. locustae*

The trophozoites of *M. locustae* appeared to be extracellular parasites of the epithelia of the Malpighian tubules (Fig. 2), midgut and gastric caeca. Histopathology in these tissues of grasshoppers containing few trophozoites and cysts were not evident because: (a) spent epithelial cells of the midgut and caeca normally are replaced by cells from the regenerative nidi (Day and Waterhouse, 1953), (b) other nonsusceptible tissues, such as fat bodies, pericardial cells, and the hindgut, supplement the excretory activity of the Malpighian tubules (Patton, 1953), and (c) infections by *M. locustae* apparently initiated increased proliferation of Malpighian tubules. It was noted that the tubules of infected grasshoppers were unusually prominent and often covered and obliterated the midgut when dissected grasshoppers were examined with a dissecting microscope.

The histopathology observed in heavily infected grasshoppers indicated that the susceptible tissues were functionally impaired. As an example, in some sections of heavily infected midguts and gastric caeca pieces of epithelial tissues were disconnected from the basal membrane and showed indications of having been sloughed prior to being replaced by regenerative cells (Fig. 3). The sloughed tissues contained trophozoites and cysts of *M. locustae*. It was considered that either the nidi were infected, which has not been established, or the tissues were sloughed more rapidly than could be replaced by regenerative cells. Such heavily infected midguts appeared flaccid and occasionally exhibited lesions which apparently facilitated lethal bacterial infections in the hemocoel.

Heavily infected grasshoppers lacked peritrophic membranes which, according to Day and Waterhouse (1963), protects the epithelium of the midgut from coarse particles of food.

Heavily infected Malpighian tubules, which were devoid of epithelial tissues, usually appeared about 4 times their normal diameter due to the pressure exerted by heavy accumulations of cysts (Fig. 4). Increased localized expansion of these compacted tubules in Schistocerca vaga vaga produced white nodules, up to 3 mm in diameter, which were packed with cysts. Occasionally cysts were observed in the hemocoel which apparently resulted from the rupture of the compacted tubules. Cystocytic clumping around the compacted tubules produced inflammatory growths which encapsulated the tubules and freed cysts (Fig. 5).

Secondary histopathology also was observed in tissues which were not infected with trophozoites of the amoeba. During infection the nuclei of the muscles changed from the normal ellipsoidal shapes with dispersed chromatin material to hypertrophied spherical forms in which the intensely staining chromatin material condensed peripherally inside the nuclear membrane. These pycnotic nuclei atrophied and some were observed as free floating bodies which indicated that they had been sloughed. During these nuclear alterations the muscle fibers around the degenerate nuclei appeared increasingly granular and lacked the normal striated appearance. Some muscle fibers of grasshoppers in advanced or terminal stages of infection appeared granular throughout, were atrophied and were lined with pericardial cells and spheroid hemocytes.

Gross observations of dissected grasshoppers indicated that infected grasshoppers contained less fat body tissue than uninfected grasshoppers. Pycnotic and free floating atrophied nuclei also were observed during microscopic examinations of fat tissues from heavily infected grasshoppers.

These secondary effects in nonsusceptible tissues probably resulted from toxic materials which accumulated in the hemocoel from the malfunctioning of the Malpighian tubules and midgut. In line with this, Prinsloo (1961b, 1962) isolated an antibiotic factor from infected Malpighian tubules of Locusta pardalina which in addition to being active against Pseudomonas aeruginosa Migula and a species of Klebsiella Trevisan also was lethal to locust embryos when added to a saline-Vitamin B-complex medium which was used to maintain the embryos.

In a histochemical study of the fat bodies of infected females and fatty acids in eggs from infected females Jackson et al. (1968) found that although infected females had less fat bodies than uninfected grasshoppers, the total lipid content in the eggs from the two groups was the same. This indicated that the female's metabolism operated sacrificially in maintaining egg lipid requirements. However, these authors also reported that the relative amounts of unsaturated fatty acids differed in eggs from infected and uninfected females and they postulated that these differences may contribute to the decreased viability of eggs from infected females. Prinsloo (1961a, b) reported that the reduced viability of the eggs and offspring from infected Locusta pardalina was due to the production of the antibiotic factor in infected Malpighian tubules. She

reported that this factor inhibited the growth of a species of Klebsiella which forms the normal flora of the ovaries and that the destruction of these flora changed the diapause sequence in the eggs.

According to Venter (1966) virtually no egg development occurred in the females in a field population of L. pardalina in which there was a high incidence of infections by M. locustae. He suggested that this prevented an expected outbreak of locusts in the area occupied by this population. According to Henry (1968), reduced fecundity and decreased egg viability were observed during an attempt to establish a laboratory culture of Schistocerca vaga vaga. The culture was started with about 60 field-collected grasshoppers which produced about 4000 grasshoppers for the first laboratory generation. Virtually all the adults of the first generation were heavily infected with M. locustae. Both fecundity and egg viability declined steadily during the following generations and the culture was nearly lost during the fifth generation. Following the advent of procedures for controlling M. locustae, no difficulty was encountered in maintaining S. vaga vaga in the laboratory.

Transmission of M. locustae

Because of infections in the midgut, gastric caeca and Malpighian tubules, cysts of M. locustae were released with the feces of grasshoppers. Based on the results of studies in which the number of cysts in feces were counted, it was determined that chronically infected adults of M. sanguinipes, M. bivittatus, and S. vaga vaga normally eliminated about 10^5 to 10^6 cysts per mg of fecal material. In some instances severely infected

grasshoppers eliminated as many as 10^8 to 10^9 cysts per mg of feces and occasionally eliminated white fecal pellets which consisted almost entirely of cysts. Because of such a high rate of release of cysts, fecal contamination appeared to be the most prominent way in which M. locustae is transmitted. In connection with this, Dr. Peggy Ellis (personal communication, 1968) mentioned that the air in the locust breeding rooms was seriously contaminated with cysts which resulted in a great deal of cross-infections between cages of grasshoppers.

Cysts of M. locustae were observed in the froth of egg pods deposited by infected females. Since neither cysts nor trophozoites have been observed in either the organs of the internal genitalia or in squashed preparations of dechorionated eggs, it appeared probable that contamination of the egg pods resulted from cysts being eliminated with the feces and remaining attached to the appendages of the external genitalia and then being passively incorporated into the egg pods during oviposition. This may have accounted for infections among nymphs which had been transferred immediately after hatching and reared under clean conditions.

Control of M. locustae in Grasshoppers in Laboratory Cultures

Various techniques have been used to control M. locustae in grasshoppers in laboratory cultures. Dr. Peggy Ellis (personal communication, 1968) reported that infestations worsened during periods of lax care and hygiene in the locust rearing rooms. She mentioned that 1% formalin solutions or an iodoform disinfectant were effective in disinfecting cages and sand. According to Cowan (1966) cleanliness was important in the

control of protozoans in grasshopper cultures. He reported that plastic rearing tubes and cages were washed in 10% Chlorox^{8/} solutions, after which specially designed metal cages were sterilized by dry heat.

Henry (1968) conducted 2 tests in which the concentrations of cysts in feces and grasshoppers were reduced by feeding and injecting various sulfonamide antibiotics. It appeared that the antibiotics had inhibited the action of the trophozoites, but because of high pretreatment accumulations, cysts continued to be eliminated for prolonged periods after the start of treatments. However, relatively high dosages of the antibiotics (10 to 50%) were used in these tests and since there were no gross differences between the treated and untreated grasshoppers, the high dosages were considered as detrimental to the grasshoppers as were infections by M. locustae.

In a third test (Henry, 1968) a triple sulfa antibiotic solution consisting of sulfamethazine (6%), sulfathiozole sodium (12%) and sulfa-pyridine sodium (8%) was added to the artificial diet at concentrations of 1.0% and 0.1% and was tested as a prophylactic control for M. locustae. In this test nymphs were fed the diets from hatching through the testing period and were challenged with cysts when 4 days old and again when 6 days old. They possibly also were challenged by contaminating cysts in that they were purposely reared close to infected grasshoppers. None of the grasshoppers fed either one of the antibiotic media were infected while all the control grasshoppers fed the medium without antibiotics were infected. More recent observations have indicated that the artificial diet alone was not very satisfactory for grasshopper growth and

development. Since then, this medium containing a 0.5% concentration of the triple sulfa solution has been used along with fresh lettuce and wheat bran for feeding grasshoppers. This method of administering the antibiotic apparently has been effective in maintaining M. locustae at low incidence.

Studies were not conducted on determining the action of the triple sulfa solution on M. locustae, nor were tests conducted on the viability of cysts released by antibiotically-treated grasshoppers. However, the antibiotic also inhibited the growth of other protozoans, particularly eugregarines, and influenced infections by the GIBV. For this reason, grasshoppers destined for studies involving these organisms were reared individually and checked by fecal examinations for infections by M. locustae prior to use.

Eugregarinida

According to the classification of Honigberg et al. (1964) the subclass Gregarina Dufour contains three orders of gregarines. Two orders, Eugregarinida Legér and Neogregarinida Grassé, include species which are pathogenic in grasshoppers. The Eugregarinida were divided further into the suborder Acephalina von Kolliker, in which the trophozoites are nonseptate, and the suborder Cephalina Delage and Herovard, in which the trophozoites are septate and usually consist of three segments. Only cephaline-type eugregarines have been reported from grasshoppers and locusts.

Differentiation of Eugregarinida from Grasshoppers

Both Watson (1916) and Semans (1936, 1939, 1941, 1943) presented comprehensive reports on the eugregarines of grasshoppers and locusts. However, these authors reported that some species which they listed probably were not valid because the descriptions were based on very limited material, which in some instances was a single trophozoite. Also, many descriptions were based on highly variable characteristics, such as the ratios of the length to the width of the body segments. More recent descriptions of previously undescribed species usually included morphological descriptions of cysts, spores and trophozoites.

Numerous eugregarines were isolated from grasshoppers during the present studies and attempts were made to differentiate them according to existing methodology. However, it was found that the trophozoites and cysts frequently varied so much in size and shape that the eugregarines under study could be assigned to any one of several described species. For example, the variations in the appearance and measurements of trophozoites and cysts from one Melanoplus bivittatus were such that these gregarines were either Gregarina paracoblattae Semans, Gregarina rigida columna Semans, or Actinocephalus elongatus Semans. In another case the measurements of cysts from six species of grasshoppers from one location in Montana formed a trimodal distribution when plotted which indicated the possibility of three species of gregarines (Fig. 6). However, because of the large overlap in each peak distribution, it was not possible to assign any of the cysts to a particular species. It was noted that the cysts from the predominate grasshopper at this location, Melanoplus bruneri,

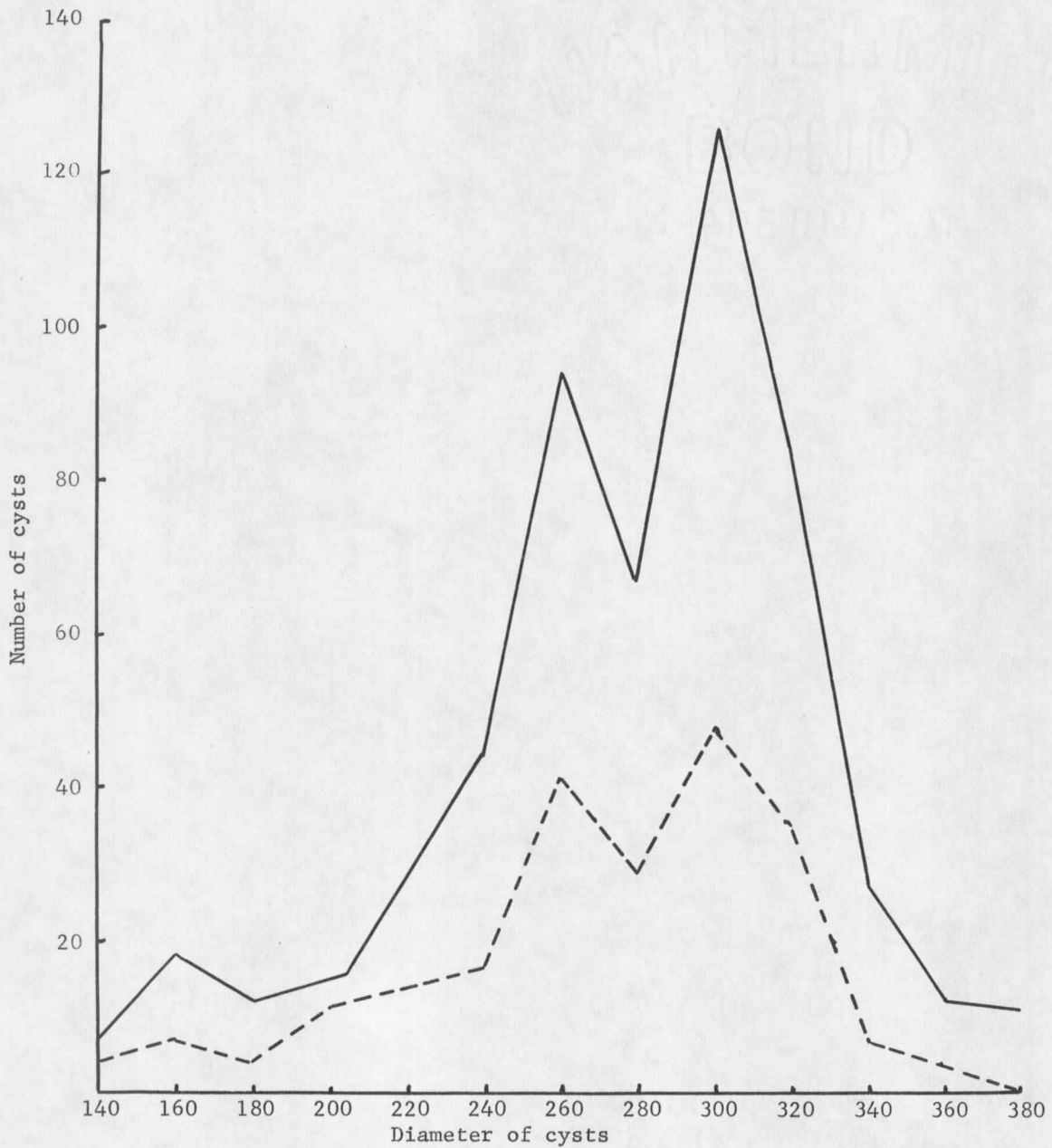


Fig. 6.--Distributions of measurements of cysts (in microns) of Eugregarinida from Melanoplus bruneri, Melanoplus oregonensis, Bruneria brunnea, Camnula pellucida, Chorthippus curtippennis, and Neopodismopsis abdominalis (solid line) and Melanoplus bruneri alone (dashed line) which were collected in the Antelope Basin area of southwestern Montana.

also followed the same trimodal distribution.

Host and Geographical Ranges of Eugregarinida in Grasshoppers

Watson (1916) listed 25 species of grasshoppers from Europe and North and South America as hosts of various eugregarines while Semans (1941) listed 39 host species from 4 subfamilies of Acrididae. Some eugregarines listed by Semans showed both wide host and wide geographical ranges. For example, Gregarina nigra Watson was reported from grasshoppers from South Africa and the United States, and Gregarina rigida rigida (Hall) was reported from 15 host species throughout the United States. Canning (1956) described Gregarina garnhami Canning from Schistocerca gregaria Forsk. and Locusta migratoria migratorioides R. & F. in laboratory cultures in England and from S. gregaria and Anacrydium aegyptium Linn. which were collected in Egypt. According to Canning, G. garnhami resembled the New World species of G. rigida rigida more closely than a species, Gregarina oblonga Dufour, which had been described from L. migratoria and the cricket Gryllus campestris from France.

During the present studies eugregarines were observed in Melanoplus sanguinipes, M. bivittatus, M. differentialis, M. femurrubrum, M. packardi, M. bruneri, M. dawsoni, Melanoplus alpinus Scudder, Melanoplus oregonensis (Thomas), Melanoplus borealis borealis (Fieber), Aulocara elliotti, Phoetaliotes nebrascensis (Thomas), Psoloessa delicatula, Bruneria brunnea (Thomas), Chortophaga viridifasciatus (DeGeer), Camnula pellucida (Scudder), Arphia conspersa Scudder, Arphia pseudonietana pseudonietana (Thomas), Xanthippus corallipes, Hadrotettix trifasciatus, Chorthippus curtipennis,

Encoptolophus sordidus costalis (Scudder), and Neopodismopsis abdominalis (Thomas) from Montana; Melanoplus sanguinipes, M. bivittatus and Oedaleonotus enigma (Scudder) from Idaho; and M. sanguinipes, M. cuneatus, A. ellioti, P. delicatula and H. trifasciatus from Arizona.

Generalized Life Cycle of Eugregarinida in Grasshoppers

Figure 7 shows a diagrammatic representation of the generalized life cycle of eugregarines as determined by the stages observed during the present studies. The spores are ingested and germinate in response to some stimulus in the digestive tract. The released sporozoites attach to the epithelial layers of the midgut and gastric caeca and eventually develop into cephalonts with three body segments. After sloughing the apical segment, epimerite, the resulting sporonts move freely in the digestive tract lumen. The sporonts then associate into pairs in a linear arrangement and undergo a series of revolving movements during which the free ends also become attached to form a gametocyst. The cyst continues revolving and secretes an external gelatinous membrane. The nucleus and septum dividing the protomerite and deutomerite of each gametocyte disappear during the transformation into a cyst. Small spherical gametes form within each gametocyte after which the membrane separating the gametocytes disintegrates and the internal structure of the cyst becomes uniformly granular. Apparently two gametes then fuse to form a zygote which then transforms into a spore. During this process, small basal discs develop on the cyst wall from which invaginated spore ducts develop. At maturation the spore ducts are extruded and a long filament of spores is

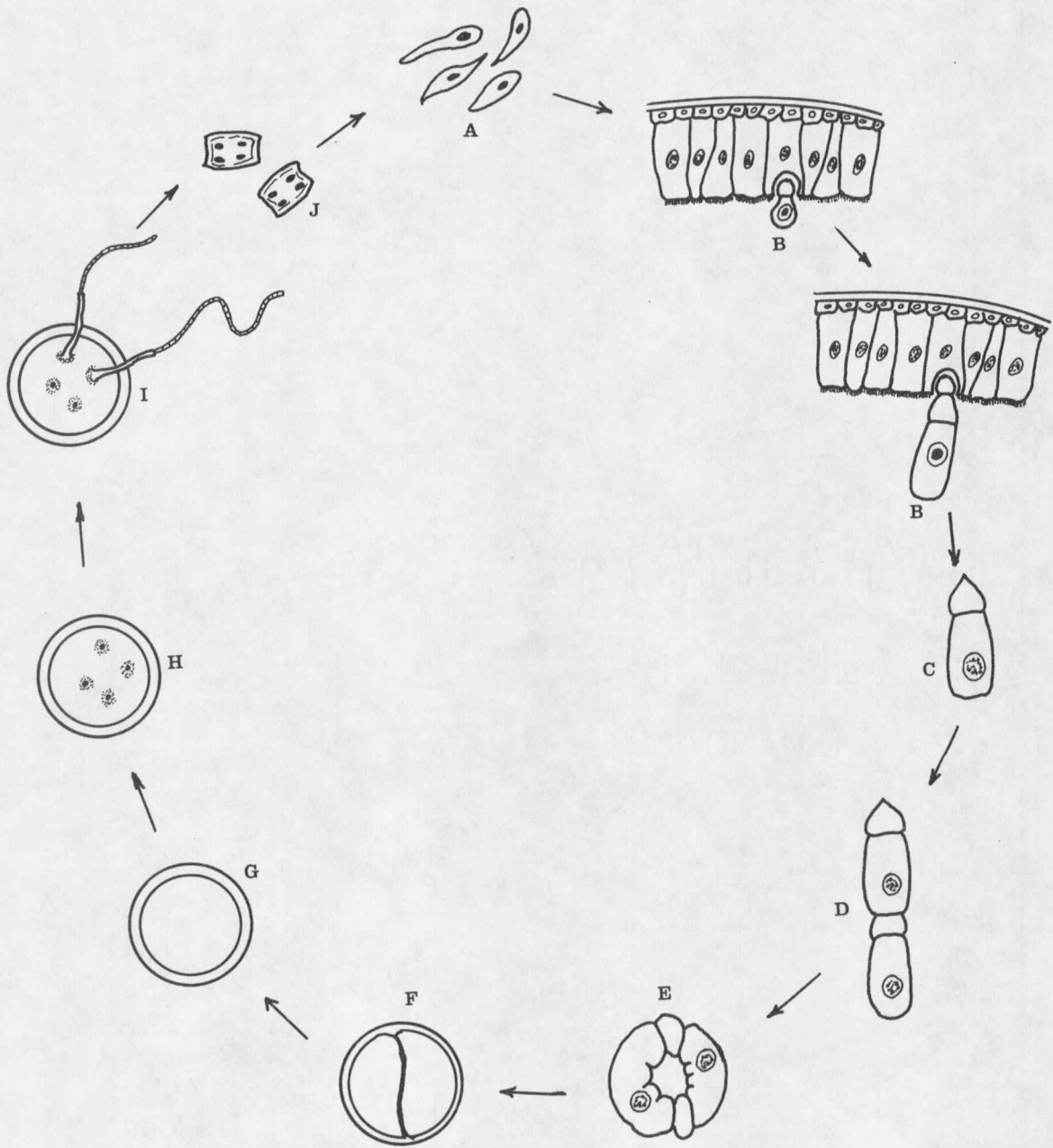


Fig. 7.--Diagrammatic representation of the life cycle of Eugregariniida as determined from stages observed during studies of infections in grasshoppers. A, sporozoites; B, cephalonts; C, sporont; D, an association between two sporonts; E, appearance of an association during encystment; F, a gametocyst with inter-gametocyte membrane; G, cyst; H, cyst with basal discs; I, cyst with two extruded spore ducts and spore filaments; J, spores.

expelled through each duct.

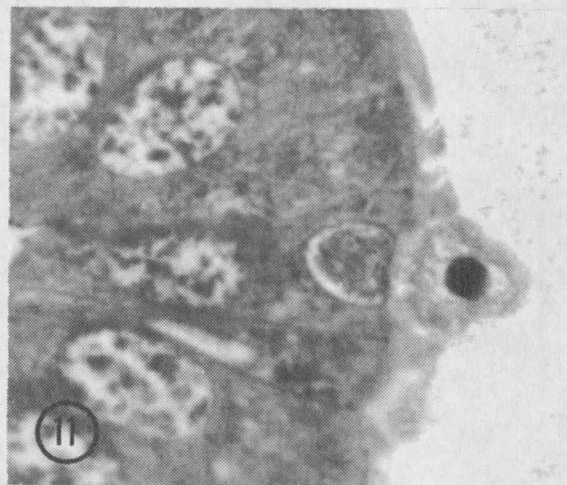
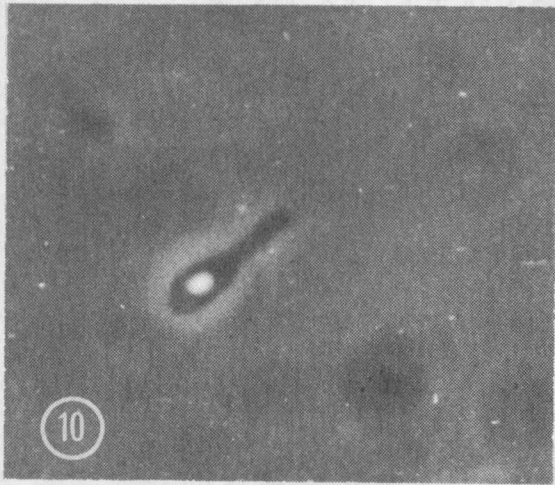
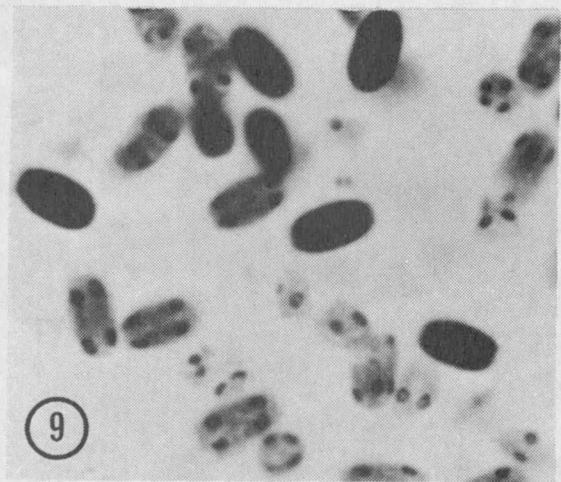
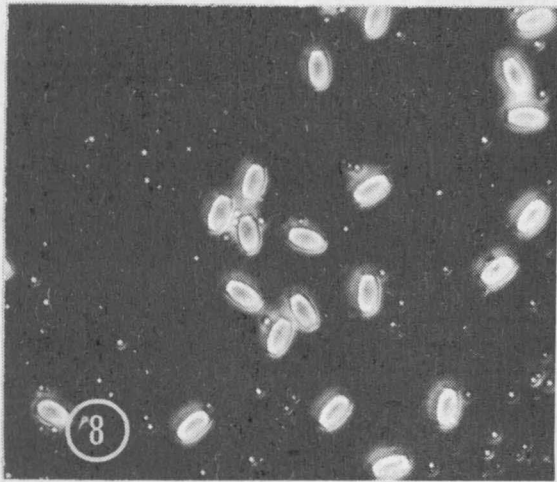
Stages in The Life Cycle of Eugregarines in Grasshoppers

Extruded spores appeared ellipsoidal with truncate ends and measured about 7.0 microns long by 5.3 microns in diameter (Fig. 8). They possessed a thick wall which appeared light refractive under phase contrast microscopy. Truncate spores in long filaments have been observed in squash preparations of cysts which indicated that the filaments form prior to extrusion. Immature spores without truncate ends were present in the squash preparations of cysts.

According to Canning (1956) each spore of Gregarina garnhami contained eight sporozoites which were arranged such that the enlarged nuclear ends of four were at each end of the spore. However, Harry (1965) showed spores of G. garnhami which contained spirally arranged sporozoites. Allegre (1948) reported that the spores of G. rigida contained eight sporozoites which were spirally arranged and that freed sporozoites appeared cylindrical with tapered ends and measured 7.3 microns long by 1.2 microns in diameter.

On several occasions during the present studies mature cysts were squashed and the smears were fixed and stained to determine the nuclear arrangement with zygotes and spores. In all cases there appeared to be eight nuclei within each spore (Fig. 9) which were arranged with four at each pole. However, the sporozoites from single spores observed in fresh preparations, were variable in size and shape, some oval, tapered at both ends, tapered at only one end or tadpole-shaped (Fig. 10). They usually

- Fig. 8. Mature spores extruded from a cyst of *Eugregarinida* from a grasshopper. 700X.
- Fig. 9. Sporoblasts from a cyst of *Eugregarinida* from a grasshopper. Sectioned preparation fixed in Gilson's fluid and stained with iron hematoxylin and eosin. 1,400X.
- Fig. 10. Fresh preparation under phase contrast microscopy of a sporozoite which was extruded from a spore of *Eugregarinida* from a grasshopper. 5000X.
- Fig. 11. Young cephalont of a *Eugregarinida* with the epimerite segment embedded in the cytoplasm of an epithelial cell of a gastric caecum of *Melanoplus sanguinipes*. Sectioned preparation fixed in Gilson's fluid and stained with iron hematoxylin and eosin. 620X.
- Fig. 12. Fresh preparation under phase contrast microscopy showing a group of cephalonts of *Eugregarinida* in tissues of a gastric caecum of a grasshopper. 100X.



measured from 4 to 5 microns in length by about 1 to 2 microns in diameter. Each contained a light refractive granular structure which was probably the nucleus. The sporozoites disintegrated within one hour after extrusion in distilled water.

Harry (1965) reported that spherical sporozoites of G. garnhami remained free in the host's digestive tract for 12 hours before they attached to the epithelium. During the present studies attached sporozoites were observed which were partially embedded in the epithelial cell and initial growth was evident from the increased size of the embedded portion and increased size of the nucleus which was outside the cell margin. As result, the young cephalont acquired a dumbbell shape which persisted until the embedded structure, epimerite, was 13 to 16 microns in diameter. By the time the exposed posterior portion, deutomerite, was about 20 microns in length, the protomerite began diametric development as an annulus between the epimerite and deutomerite. The 3 segments were discernible by the time the cephalont was about 50 microns long (Fig. 11).

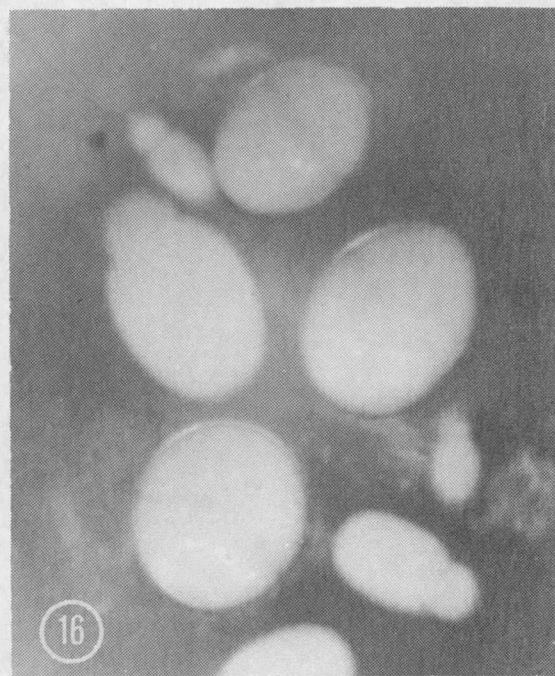
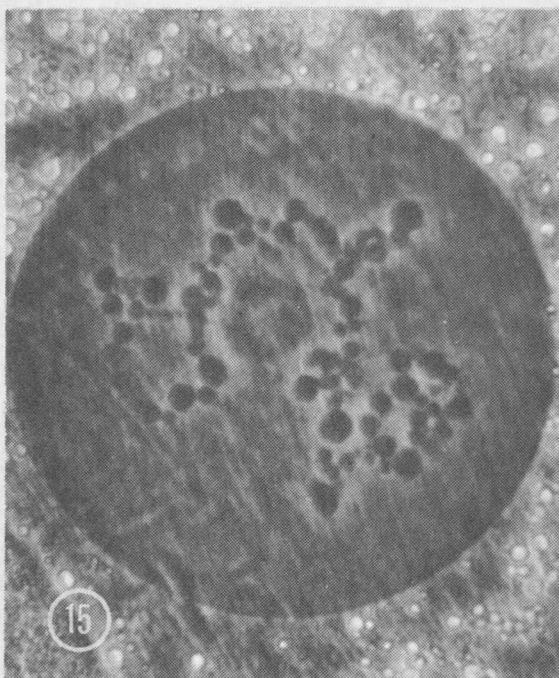
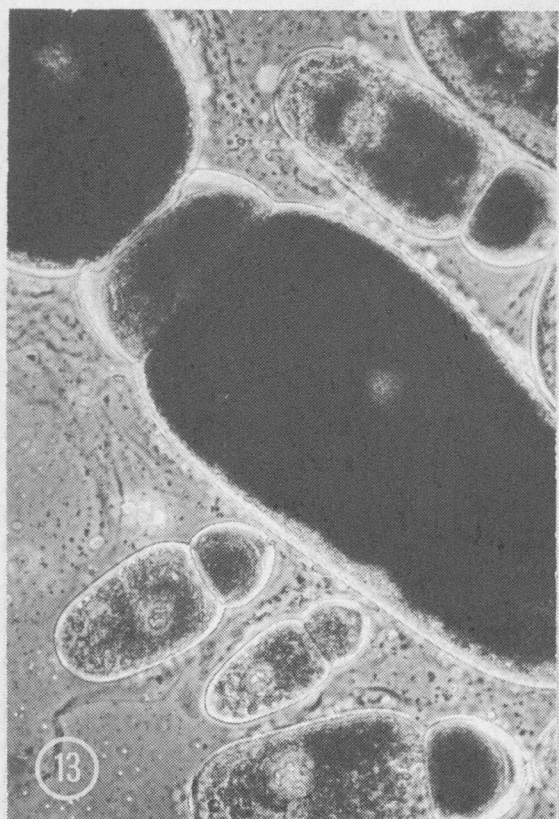
Because the sporozoites only partially penetrated the epithelial cells Harry (1965) reported that gregarines could not be considered as intracellular parasites. He suggested that the intracellular structures observed by Allegre (1948) actually were degenerative products which occur normally during the host's molting cycle. However, intracellular structures were observed during these studies which appeared to be young cephalonts and not degenerative products because they were not present in other epithelial sections.

While still attached to the epithelium the cephalonts usually continued growth up to about 300 microns in length by about 100 microns in diameter (Fig. 12). The cephalonts then sloughed the epimerite segment and became free moving trophozoites (sporonts) in the lumen of the digestive tract (Fig. 13). Although the stimulus for detaching trophozoites has not been determined, the usual presence of small sporonts, which were less than 100 microns in total length, indicated that detachment was not dependent on the size of the trophozoite. According to Canning (1956) the trophozoites of G. garnhami continued to grow after detachment.

The sporonts migrated to and accumulated in the lumen of the anterior part of the midgut. Microscopic examinations of sporonts in fresh preparations indicated that they moved on a mucous layer which was secreted through the body wall. Beams et al. (1959) observed that the body wall of G. garnhami consisted of 3 membranes which were porous or which contained tubular structures through which the mucous substances were secreted. They also observed myonemes in the body wall which they suggested were responsible for the contraction and bending of the body during movement.

Paired sporonts (Fig. 14) most frequently occurred in the anterior part of the midgut. However, they have been observed in caeca and also some sporonts were joined to cephalonts which were still attached to the epithelial cell. Usually these associations were limited to two sporonts: an anterior primite and a posterior satellite. Occasional associations

- Fig. 13. Various sized sporonts around the satellite of an association of Eugregarinida from the lumen of the midgut of a grasshopper. Fresh preparation under phase contrast microscopy. 200X.
- Fig. 14. An association consisting of an anterior sporont (primita) and a posterior sporont (satellite). Fresh preparation under bright light microscopy. 150X.
- Fig. 15. Fresh preparation under phase contrast microscopy of the nucleus of a sporont of Eugregarinida showing the beaded arrangement of the karyosomes around a nucleolus. 1500X.
- Fig. 16. Sporonts and newly formed gametocysts of Eugregarinida from a Melanoplus sanguinipes. 100X.



have been observed between 3 sporonts, a large primitive with 2 small satellites attached parallel to each other, or 3 or more sporonts all of about equal size and arranged in a linear sequence. Although the stimulus and mechanism of attachment has not been determined, Canning (1956) observed that the presumptive satellites possessed an indentation at the anterior end of the protomerite segment which was functional in attachment. Although not expressed by Canning, this structural difference between presumptive primitives and satellites might represent sexual dimorphism in eugregarines.

The size of associations varied both within individual grasshoppers and between grasshoppers. For example, the lengths of ten associations from a male M. sanguinipes varied from 548 microns to 833 microns, with an average length of 690 microns. The lengths of the primitives in these associations varied from 274 to 363 microns, with an average of 338 microns, while the satellites measured from 264 to 412 microns with an average of 355 microns. In seven associations the satellite was longer than the primitive. In contrast, the associations from a M. bivittatus, which was collected at the same location as the M. sanguinipes above, averaged about 900 microns in length, but the primitives averaged 678 microns and the satellites averaged 211 microns in length. In another case the associations from M. bivittatus averaged 899 microns in total length while those from M. sanguinipes, which were collected at the same time and at the same location, averaged 314 microns in length. Although the possibility exists that these size differences indicated specific

differences, it appeared more probable that they were due to differences in hosts. Although not fully investigated, similar size differences in association also have been evident in the different sexes of species in which the female is considerably larger than the male, such as Chorthippus curtipennis, Gamnula pellucida, and Aulocara ellioti. Apparently Canning (1956) observed similar inconsistencies in work with G. garnhami since she reported that the ratios of measurements and absolute dimensions were not taxonomically significant and that ratios of measurements for specific diagnosis should be used with discretion.

The nucleus was always located in the deutomerite and, in larger sporonts measured up to 50 microns in diameter. The nuclei contained karyosomes which were variable in structure. Cephalonts generally contained a single karyosome which appeared as a large sphere located centrally in the nucleus while in sporonts the karyosomes were irregular in numbers, size and shape. In Fig. 15 is shown a nucleus of a sporont in which the karyosomes appeared in a beaded arrangement. Generally the nucleolus appeared as a small dense sphere in the central area of the nucleus. The irregularities in karyosome structure suggested that they undergo cyclic changes during the development of the eugregarine and particularly during the formation of the associations.

The gametocysts, which were spherical and white to orange in color (Fig. 16), developed by the associations moving in a circular or rotating pattern during which the free ends of the association became connected. During these movements the gametocytes secreted the mucous substance which

formed the cyst wall. Rotational movements apparently continued until the increased thickness of the wall inhibited further movements. According to Canning (1956) the transformation of associations into cysts took about 2.5 hours which corresponded to observations made during the present studies. Allegre (1948) reported that cysts of G. rigida were present at 4 days after the appearance of the first associations and about 12 to 14 days after infection. During the present studies cysts were usually present 8 days after experimental infection.

Both the nuclei and septa between the protomerite and deutomerite of the gametocytes disappeared during formation of the gametocysts while the membrane separating the gametocytes disappeared within 24 hours after encystment. The opaque spots (basal discs) appeared on the cyst wall at about 48 hours after encystment. Eventually these spots, which appeared variable in numbers, changed to an orange color with the formation of spore ducts. The newly formed spore ducts extended internally into the cyst and were extruded just prior to the release of spores.

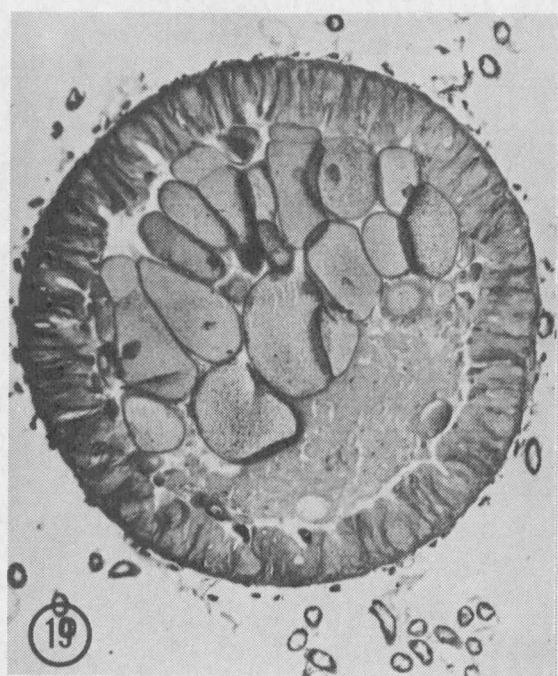
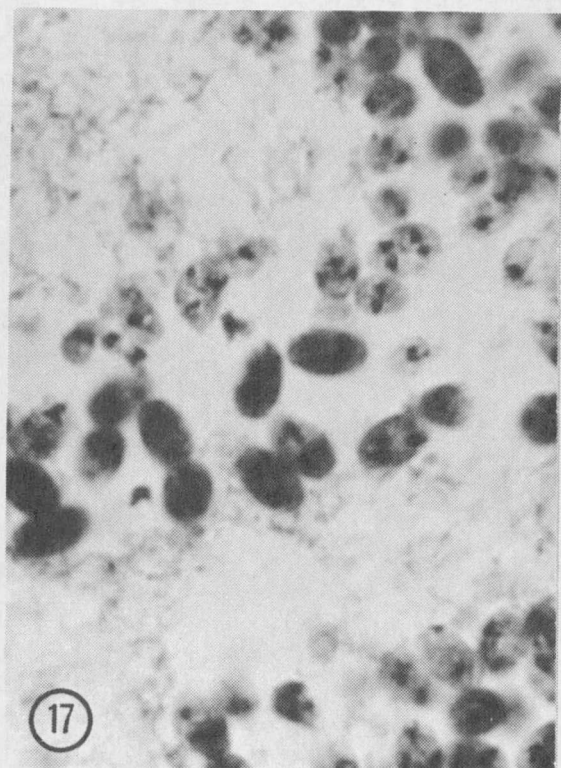
Gametes usually were evident in each gametocyte prior to the disappearance of the intergametocyte membrane. There appeared to be a mixing of the internal structures within the cyst after the disintegration of this membrane and it was probable that the gametes of one gametocyte fused with the gametes of the other gametocyte to form zygotes. What were considered as zygotes appeared as spherical bodies, about 6 microns in diameter, with one to several darkly staining nuclei (Fig. 17). Eventually the zygotes developed into elongate, spore-shaped bodies with

Fig. 17. Newly formed sporoblasts in a sectioned preparation of a cyst of *Eugregarinida* from grasshoppers. 1350X.

Fig. 18. Cross section of a gastric caecum from a *Melanoplus bruneri* showing cephalonts of *Eugregarinida* attached to the epithelium. Preparation fixed in Gilson's fluid and stained with alum hematoxylin and eosin. 100X.

Fig. 19. Cross section of the anterior part of the midgut of a *Melanoplus bruneri* in which the lumen is nearly filled with trophozoites of *Eugregarinida*. 53X.

Fig. 20. Fecal pellets from a *Melanoplus sanguinipes* with attached cysts of *Eugregarinida*. 25X.



eight darkly staining nuclei (Fig. 9). Truncate spores were observed in cysts which were examined prior to extrusion of the spore ducts. They were arranged in chains and each chain was enclosed in a thin gelatinous tube. Extruded spores also were arranged in chains within the gelatinous tubes.

According to Canning (1956) the interior of cysts of G. garnhami varied from 114 to 470 microns in diameter. She presented data which showed that with the gelatinous walls included the cysts measured from 137 to about 500 microns in diameter. Allegre (1948) reported that the cysts of G. rigida varied from 212 to 505 microns in diameter and that the size of cysts increased with the development of the host. For example, the cysts from 12-day old M. differentialis averaged 362 microns in diameter, and those from adult grasshoppers averaged 444 microns in diameter.

Similar variations in cyst measurements were noted during the present studies and particularly in the measurements of cysts from 6 species of grasshoppers, Melanoplus bruneri, M. oregonensis, Camnula pellucida, Chorthippus curtipennis, Bruneria brunnea, and Neopodismopsis abdominalis, which were collected at a site in southwestern Montana. As shown in Fig. 6, the distribution of cyst measurements when plotted resulted in three peak sizes, one at about 160 microns, one at about 260 microns, and one at about 300 microns. It was considered possible that the two larger peaks represented two species of gregarines; however, because of the amount of overlap in the peak distributions and the lack of additional

differences in the trophozoites and spores, specific differentiation could not be established. Instead, it appeared that the peak at 260 microns was derived from cysts from male grasshoppers, and the peak at 300 microns from cysts from females. This was evident in the measurements of cysts from N. abdominalis, in which only three females were infected. All but three of 33 cysts from these females measured between 280 to 340 microns; the remaining three cysts measured 220 microns or less. In addition, the measurements of cysts from the predominant grasshopper at this site, M. bruneri, followed a similar size distribution which indicated that the two larger peaks resulted from cysts from each of the sexes.

From observing the development of these cysts it was determined that the small cysts, about 200 microns or less, were not viable. Apparently such cysts resulted from the encystment of individual sporonts, which has been observed, or from associations in which one gametocyte was unusually small, usually the satellite, which also has been observed. This indicated that recombination between mature sporonts is necessary for continued development.

Histopathology of Infections by Eugregarinida in Grasshoppers

Kamm (1920) reported that epithelial cells to which cephalonts of G. rigida were attached showed no evidence of injury. Similarly Ganning (1956) reported that G. garnhami caused little or no damage to locusts and concluded that because epithelial cells were periodically replaced eugregarines were relatively harmless to their hosts. After having examined numerous sections of midguts and gastric caeca of heavily infected

grasshoppers, it was concluded from the present studies, however, that eugregarines are detrimental to their hosts. For example, partial destruction of the epithelium was observed in some sections of gastric caeca (Fig. 18). The peritrophic membranes were usually lacking from the midgut lumens of heavily infected grasshoppers. As a result the epithelial tissues appeared more susceptible to damage from coarse food particles. In addition, sections have been observed in which the lumen of the midgut was nearly filled with trophozoites (Fig. 19). Obviously such concentrations of eugregarines inhibited the movement of food materials through the digestive tract. Because of the lack of reliable techniques for quantitative experimental infections, studies have not been conducted on the effect of infections by eugregarines on the reproduction of grasshoppers.

Source of Infections by Eugregarinida in Grasshoppers

Since cysts were eliminated with feces (Fig. 20) the main source of infections appeared to be from contaminated food. It was observed that the gelatinous tubes containing spores were sticky and it appeared because of this that the spores would easily adhere to the appendages of grasshoppers.

In experimental infections of grasshoppers in which the eliminated cysts were removed from the rearing tubes, the gregarines usually underwent a single cycle of infection and all cysts were eliminated during a period of less than 10 days. However, several cases were observed in which the grasshoppers continued to eliminate cysts over a prolonged

period. As an example, 673 cysts were recovered during a 21-day period from the feces of an experimentally infected M. bivittatus which had been individually reared. This prolonged release of cysts indicated that reinfection had occurred by autoinfection, in which case some cysts were retained in the digestive tract where they matured and released spores which then were instrumental in initiating additional infections. Empty spores were observed on several occasions in the contents of the midguts of heavily infected grasshoppers.

Neogregarinida

Steinhaus and Marsh (1962) first reported a neogregarine from grasshoppers and suggested that it belonged to the genus Gaulleryella Kielen because the sporocyst contained eight spores. Bucher (1966) apparently observed the same species and reported that the sporocysts commonly contained four to eight spores, but that a few contained nine to ten spores. Because of this Bucher postulated that the sporocysts would normally contain 16 spores which placed the neogregarine in the genus Lipotropha Keilen. Neither Bucher nor Steinhaus and Marsh observed stages of schizogony.

Host and Geographical Range of Neogregarinida in Grasshoppers

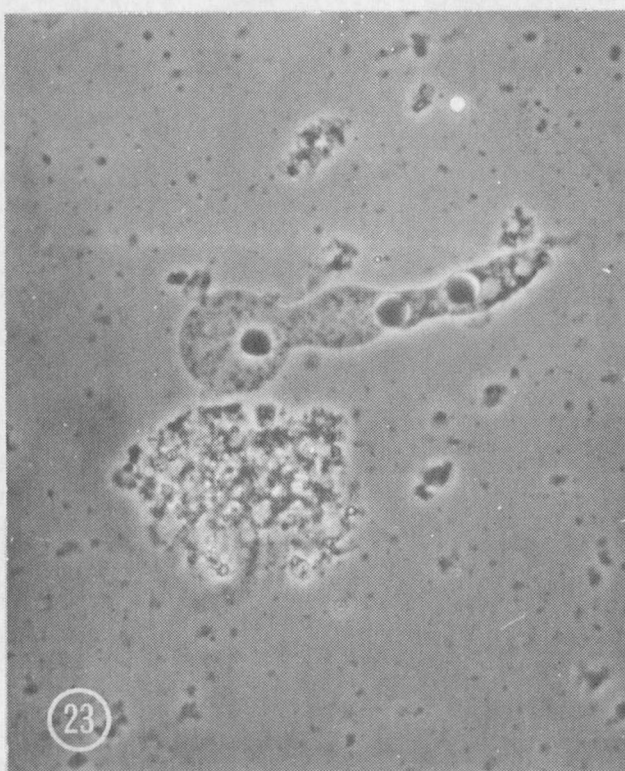
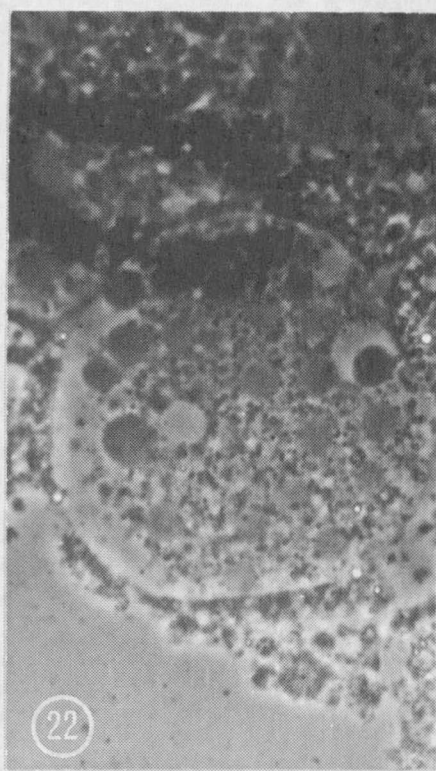
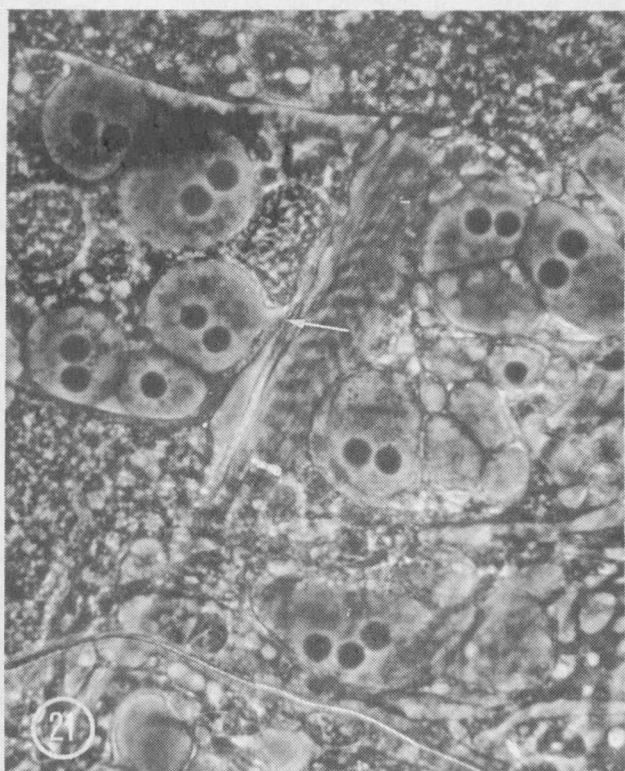
Steinhaus and Marsh (1962) observed this neogregarine in Melanoplus sanguinipes (= M. mexicanus) and in an unidentified species which were sent to them for examination by J. R. Parker, Grasshopper Investigations Laboratory, USDA, at Bozeman, Montana. Bucher (1966) observed it in

M. sanguinipes (= M. bilituratus) and M. bivittatus which were collected in Canada. During the present studies the neogregarine was isolated from M. bivittatus, M. sanguinipes, M. packardi, and Melanoplus confusus Scudder, which were collected in Montana, and from M. sanguinipes which were collected in Camas County, Idaho. Infections also were observed in M. bivittatus which were reared in the laboratory from eggs collected near Bozeman and Brady, Montana.

Stages in the Life Cycle of the Neogregarinida in Grasshoppers

Because attempts to transmit experimentally this neogregarine to uninfected grasshoppers were unsuccessful, the life cycle as reported here was based on forms observed in grasshoppers in advanced or terminal stages of infection. Efforts to extrude sporozoites from spores or to detect them in tissues also failed. What were considered to be the earliest stages observed were uninucleate schizonts which measured about 6 microns in diameter. These were observed along with other larger uninucleate and multinucleate schizonts in fresh preparations of tissues from the midgut (Fig. 21). Most of the larger schizonts were irregular in shape with a variable number of nuclei and possessed protrusions which appeared similar to the holdfasts that according to Weiser (1955) are characteristic of Caulleryella spp. Numerous other spherical forms, both uninucleate and multinucleate, were observed in the lumen of the posterior foregut, midgut, gastric caeca and, occasionally, the Malpighian tubules. The largest schizonts, which were considered as terminal stages of schizogony, measured about 50 microns in diameter and often possessed in excess of 20

- Fig. 21. Fresh preparation under phase contrast microscopy showing uninucleate, binucleate and trinucleate schizonts of the Neogregarinida of grasshoppers. One schizont exhibits a holdfast (arrow) which appears to be attached to a muscle fiber. 900X.
- Fig. 22. A terminal multinucleate schizont of the Neogregarinida of grasshoppers. Fresh preparation under phase contrast microscopy. 950X.
- Fig. 23. A vermiform trinucleate schizont of the Neogregarinida of grasshoppers which was observed in fresh preparations of tissues from the hemocoel of a grasshopper. 900X.
- Fig. 24. A granular multinuclear schizont of the Neogregarinida of grasshoppers observed in a smear preparation of the hemolymph of a grasshopper. 1100X.



nuclei (Fig. 22). These terminal stages occurred in the hemocoel along with other forms which were irregularly shaped (Fig. 23) and often appeared densely granular (Fig. 24).

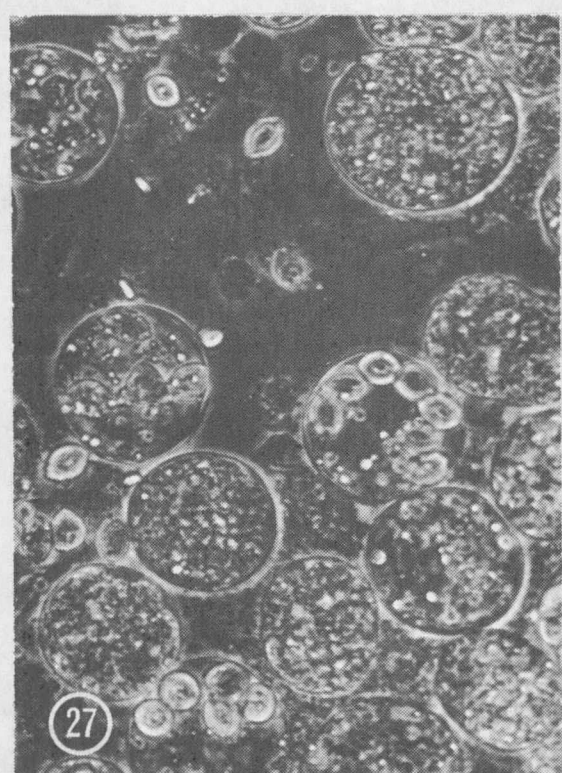
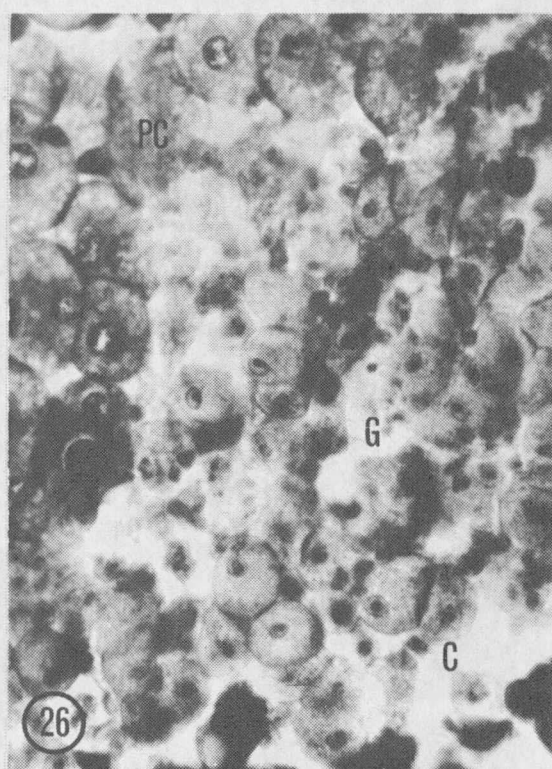
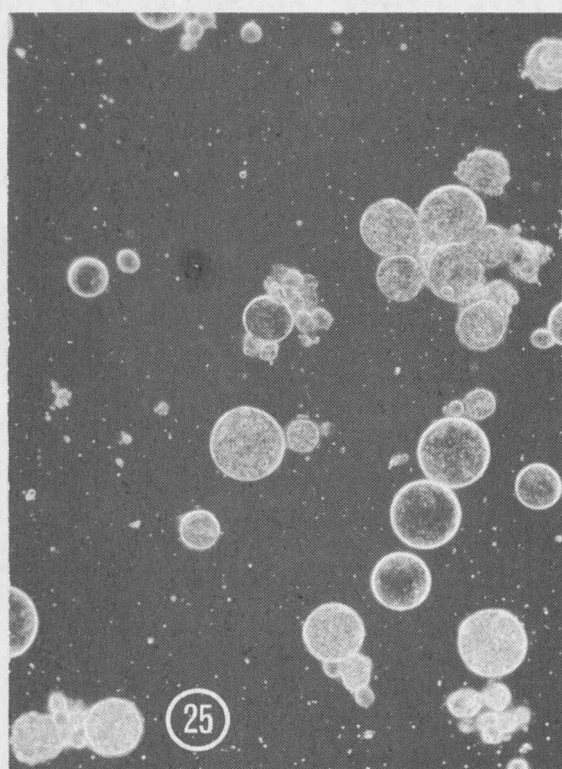
The nuclei of the schizonts in the midgut tissues usually measured about 4 microns in diameter and were visible under phase contrast microscopy. Those of the granular schizonts in the hemocoel also measured about 4 microns in diameter but were visible only in smears that had been fixed and stained (Giemsa technique). The nuclei of the terminal schizonts measured from 3 to 7 microns in diameter and apparently varied in density.

Sporogonic forms were observed in the hemocoel and were densely granular. The gametocytes were characterized as being variable in size, from 5 to 25 microns in diameter, spherical (Fig. 25), and with a single nucleus which in stained preparations measured about 5 microns in diameter. They were observed as free floating in the hemocoel or congregated with other sporogonic forms at localized infection sites.

Conjugated gametocytes were observed among the congregated sporogonic forms (Fig. 26). Each of the conjugated forms appeared hemispherically shaped with a flattened side adjacent to that of the partner. Usually a thin gelatinous membrane encircled the conjugating gametocytes.

Gametocysts also were observed among the congregated forms. They were characterized as being densely granular, about 40 microns in diameter, and either uninucleate or anucleate. It was considered that the nucleus was diffuse in the apparently anucleate forms. The gametocysts possessed

- Fig. 25. A group of granular gametocytes of the Neogregarinida observed in a fresh preparation of the hemolymph of a grasshopper. 400X.
- Fig. 26. Sectioned preparation of the fat body and pericardial tissues of a grasshopper showing a heavy concentration of gametocytes (G), some of which are conjugated (C), and host pericardial cells (PC) which show indications of deterioration. 500X.
- Fig. 27. Cysts of the Neogregarinida of grasshoppers which exhibit different stages of development. Fresh preparation under phase contrast microscopy. 900X.
- Fig. 28. Cysts of the Neogregarinida of grasshoppers which contain mature appearing spores. Fresh preparation under phase contrast microscopy. 1000X.



an external membrane which appeared transparent and slightly less than 1 micron thick.

Gametes were not observed. Instead, the granular matrix within the sporocysts differentiated into nearly spherical granular zygotes, about 7 microns in diameter, which then differentiated into spores. Differentiation of the cyst matrix into zygotes occurred progressively as evident by some cysts containing some fully formed spores, some undifferentiated zygotes, and granular matrix substance at the same time (Fig. 27). It appeared that two zygotes formed and differentiated into spores simultaneously.

Generally the sporocysts contained eight or fewer spores. A few sporocysts were observed which contained more than eight spores. However, it was not possible to determine whether the supernumerary spores were inherent or an artifact of preparation.

The spores of this neogregarine appeared navicular and measured about 7.8 microns in length by 4.5 microns in diameter (Fig. 28). Each cyst exhibited a central spherical core, measuring about 2.5 microns in diameter, which contained a number of granular objects. The spores were refractory to routinely used stains, such as Giemsa, apparently because of the thick spore wall.

Proposed Life Cycle of the Neogregarinida from Grasshoppers

Based on the developmental forms described in the previous section, the probable life cycle of this neogregarine has been diagrammatically presented in Figure 29. As indicated, the sporozoites are extruded in the

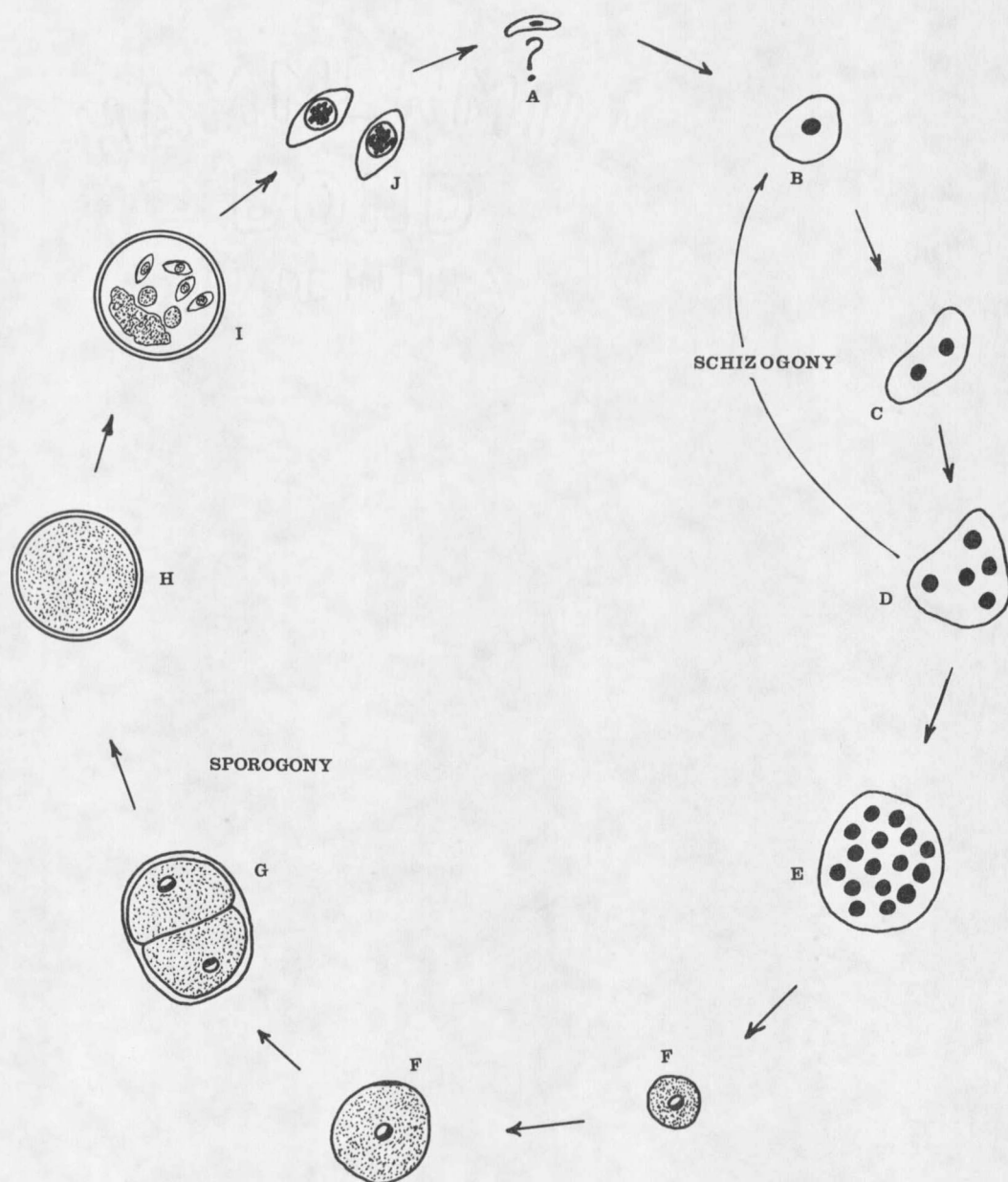


Fig. 29.--Diagrammatic representation of the proposed life cycle of the Neogregarinida from grasshoppers. A, sporozoite (not observed); B, a uninucleate schizont; C, a binucleate schizont; D, a pentanucleate schizont; E, a multinucleate terminal schizont; F, gametocytes; G, conjugating gametocytes; H, gametocyst; I, cyst with developing zygotes and spores; J, spores.

lumen of the host's digestive tract and develop into schizonts in the epithelial tissues. During schizogony, the trophozoites undergo division at irregular intervals and produce smaller trophozoites which migrate and initiate infections at other sites. Some of the trophozoites penetrate through the digestive tract wall into the hemocoel where they ultimately develop into the large terminal schizonts. The merozoites from the terminal schizonts develop into gametocytes. Fully grown gametocytes undergo sexual recombination to produce the gametocysts. The gametes from each of the gametocytes then fuse to form zygotes which then develop into spores.

The following processes in this life cycle remain to be clarified:

(1) the appearance of sporozoites and the number of sporozoites per spore, (2) the development and number of gametes per gametocyte, and (3) the development of zygotes in the cysts. However, according to the classification of Weiser (1955) the characteristics of normally eight spores per sporocyst and extracellular development, as described in the subsequent section, places this neogregarine in the genus Gaulleryella.

Histopathology of Infections by the Neogregarine from Grasshoppers

Based on the examination of sectioned tissues, the schizonts appeared to be extracellular parasites in the epithelial tissues of the gastric caeca, midgut and Malpighian tubules. As a result of these infections, the epithelia of the gastric caeca and midgut lacked the normal columnar cellular arrangement and exhibited abnormal vacuolization. There was noticeable destruction of the epithelium of Malpighian tubules in the

vicinity of the schizonts. No infections were detected in the epithelial tissues of the foregut or hindgut.

Examinations of dissected grasshoppers with a dissecting microscope showed localized intrahemocoelic inflammatory-like growths which appeared dull gray in color. Microscopic examination of these growths showed that they consisted almost entirely of sporogonic forms of the neogregarine. Based on the examination of sections of these growths and adjacent fat body tissues, it appeared that the sporogonic forms acted extracellularly in the destruction of fat body tissues (Fig. 26).

Because of the inability to transmit this neogregarine experimentally, no information has been obtained on the effect of this organism on grasshoppers. However, in collections of grasshoppers from the field, the infected grasshoppers usually died much earlier than those which showed no signs of infection. Comparing the rate of mortality of these infected grasshoppers with the rates of mortality among field collected grasshoppers which were infected with other pathogens, the virulence expressed by this organism appeared about intermediate to the other pathogens of grasshoppers. No information has been obtained on the natural means of transmission because of the inability to infect laboratory grasshoppers experimentally. There are indications that it is an obligate transovarian pathogen.

Microsporida

Three species of the Nosema Nägeli (Microsporida: Nosematidae) have been isolated from grasshoppers. Two of the species, N. locustae and

N. acridophagus, have been described. The description of the third species has been withheld pending comparison with Nosema spp. from other insects. Also several probable species of organisms were observed which were grouped under "small spore" disease-causing organisms in grasshoppers. Preliminary characterization of these organisms indicated that they probably belonged in the order Microsporida; however, further differentiation has not been possible because efforts to experimentally transmit them were unsuccessful.

Life Cycle of Nosema spp. in Grasshoppers

Except for differences in the morphology of the developmental stages, the life cycles of the Nosema spp. were very similar (Fig. 30). Each spore of Nosema spp. contains a polar filament which is extruded during sporulation. The sporoplasm (Amoebula) then emerges from the spore, either on or through the polar filament, and penetrates the tissues of the host. The amoebula, which is binucleate after emergence, develops into a uninucleate schizont. The uninucleate forms develop into binucleate schizonts which then develop into quadrinucleate schizonts. The quadrinucleate forms undergo asynchronous schizogony forming either uninucleate or binucleate schizonts which then repeat the cycle. Eventually the schizonts form diplokaryia which are characterized as binucleate forms in which the nuclei are hemispherically shaped with their flattened sides in juxtaposition with each other. The nuclei of the diplokaryon then fuse to form a sporont with a single, large and intensely staining nucleus. The nucleus of the sporont then condenses and divides to form a binucleate sporont.

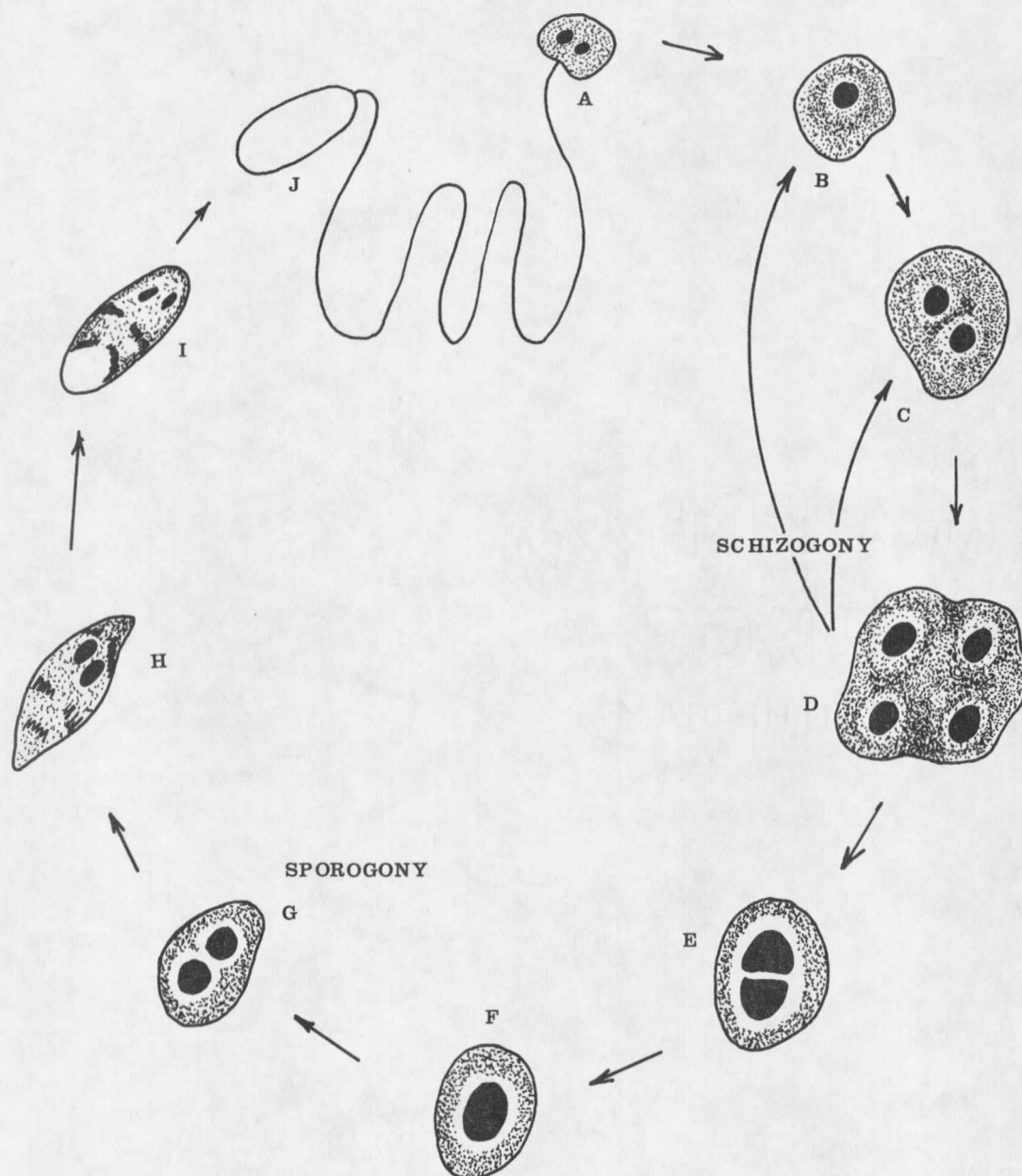


Fig. 30.--Diagrammatic representation of the generalized life cycle of *Nosema* spp. as determined from the stages observed during studies of the life cycle of *Nosema acridophagus*. A, amoebula at the end of a polar filament; B, a uninucleate schizont; C, a binucleate schizont; D, a quadri-nucleate schizont; E, a diplokaryon; F, a uninucleate sporont; G, a binucleate sporont; H, a spindle shaped sporoblast (observed only in the life cycle of *Nosema acridophagus*); I, a sporoblast; J, a mature spore with an extruded polar filament.

A sporoblast then forms from each sporont and eventually develops a light refractive spore wall and the mature spore.

Nosema locustae Canning

Host and Geographical Range of *N. locustae*

Canning (1953) described *N. locustae* from *Locusta migratoria migratorioides* in a laboratory culture in England and later (Canning, 1962) reported infections in *Schistocerca gregaria*, *Melanoplus sanguinipes* (= *M. mexicanus*), *M. bivittatus*, *M. dawsoni* and *Dissosteira carolina* (L.). Goodwin (1950, 1952) had previously reported a probable *Plistophora* sp. in *L. m. migratorioides* in Africa which according to Canning (1962) was probably *N. locustae*. Steinhaus (1951) had reported an undescribed *Nosema* in *M. bivittatus*, *M. sanguinipes* (= *M. mexicanus*) and *M. dawsoni* which had been sent to him for examination by J. R. Parker, Grasshopper Investigations Laboratory, USDA, Bozeman, Montana. In unpublished reports from the same laboratory in Montana, G. T. York (in 1952) reported the *Nosema* in *M. bivittatus* and R. E. Newton (in 1956) reported *N. locustae* from *M. bivittatus*, *M. sanguinipes* (= *M. mexicanus*), *M. bruneri* and *Aulocara elliotti*. In personal communication, G. E. Bucher of the Canadian Department of Agriculture at Belleville, Ontario, reported infections in *M. bivittatus* and *M. sanguinipes*, and R. E. Pfadt of the University of Wyoming at Laramie, Wyoming, reported a microsporidian resembling *N. locustae* in *A. elliotti* and *Cordillacris occipitalis* (Thomas).

Recently, Henry (1969a) reported that 58 species of Orthoptera were

susceptible to infections by N. locustae. In Table II are listed the 55 species of Orthoptera in which infections were observed during the present studies. The data were listed according to the source of infection: (1) occurring naturally in the field, (2) experimental application of N. locustae in the field, (3) occurring naturally in the laboratory, and (4) experimental inoculation in the laboratory.

Most of the naturally occurring infections were observed during a 6-year study of natural occurrence of N. locustae in grasshoppers in Camas County, Idaho, during which time more than 30,000 grasshoppers were examined. However, naturally occurring infections also were observed in grasshoppers from Montana, North Dakota, Minnesota, Oregon, Wyoming, Colorado, Arizona, and other areas of Idaho. The experimental infections in the field occurred in grasshoppers collected in three areas in Montana where N. locustae was applied. The infections that occurred naturally in the laboratory were observed in untreated laboratory-reared grasshoppers while the experimental laboratory infections resulted from planned inoculations.

Because N. locustae was observed in species belonging to the four traditional Nearctic subfamilies of Acrididae it was suggested that most other species of Acrididae probably are susceptible to this microsporidian. In addition to the species of Acrididae, infections also were observed in an unidentified species of Tetrigidae and the black field cricket, Gryllus sp. The spores from the black field crickets were assayed in laboratory grasshoppers and were confirmed as being N. locustae.

TABLE II

Species of Orthoptera that are susceptible to infections by N. locustae and the sources of the observed infections. (Henry, 1969a).

Host species	Source of infection ^a			
	Field		Laboratory	
	Nat	Exp	Nat	Exp
Subfamily Acridinae				
<u>Pseudopomala brachyptera</u> (Scudder)	+			
<u>Mermiria maculipennis macclungi</u> Rehn	+			
<u>Opeia obscura</u> (Thomas)	+	+		
<u>Phlibostroma quadrimaculatum</u> (Thomas)	+			
<u>Chorthippus curtippennis</u> (Harris)	+	+	+	
<u>Aeropedellus clavatus</u> (Thomas)	+			
<u>Bruneria brunnea</u> (Thomas)	+			
<u>Ageneotettix deorum deorum</u> (Scudder)	+	+		
<u>Psoloessa delicatula delicatula</u> (Scudder)	+			
<u>Aulocara ellioti</u> (Thomas)	+	+	+	+
Subfamily Oedipodinae				
<u>Arphia pseudonietana pseudonietana</u> (Thomas)		+		
<u>A. conspersa</u> Scudder	+			
<u>Camnula pellucida</u> (Scudder)	+		+	+
<u>Encoptolophus sordidus costalis</u> (Scudder)		+		
<u>Xanthippus corallipes</u> (Haldeman)	+			
<u>Cratypedes neglectus</u> (Thomas)	+			
<u>Dissosteira carolina</u> (L.)	+	+		+
<u>D. spurcata</u> Saussure	+			
<u>Trachyrhachys kiowa kiowa</u> (Thomas)	+	+		
<u>Metator pardalinus</u> (Saussure)	+			
<u>M. nevadensis</u> (Bruner)	+			
<u>Hadrotettix trifasciatus</u> (Say)	+			
<u>Trimerotropis gracilis sordida</u> Walker	+			
<u>T. pallidipennis pallidipennis</u> (Burmeister)	+			
<u>T. campestris</u> McNeill	+	+		
<u>T. laticincta</u> Saussure	+			
<u>T. fontana</u> Thomas	+			
<u>T. inconspicua</u> Bruner	+			
<u>Conozoa wallula</u> (Scudder)	+			

TABLE II, Continued

Host species	Source of infection ^a			
	Field		Laboratory	
	Nat	Exp	Nat	Exp
Subfamily Romaleinae				
<u>Brachystola magna</u> (Girard)	+			
Subfamily Cyrtacanthacridinae				
<u>Schistocerca americana</u> (Drury)			+	+
<u>S. vaga vaga</u> (Scudder)			+	+
<u>Hypochlora alba</u> (Dodge)		+		
<u>Hesperotettix viridis viridis</u> (Thomas)	+	+	+	
<u>Bradynotes obesa</u> (Thomas)	+			
<u>Phoetaliotes nebrascensis</u> (Thomas)	+	+		
<u>Oedaleonotus enigma</u> (Scudder)	+		+	
<u>Melanoplus bivittatus</u> (Say)	+	+	+	+
<u>M. dawsoni</u> (Scudder)	+	+	+	
<u>M. alpinus</u> Scudder	+			
<u>M. occidentalis occidentalis</u> (Thomas)	+			
<u>M. borealis borealis</u> (Fieber)	+			
<u>M. sanguinipes sanguinipes</u> (F.)	+	+	+	+
<u>M. bruneri</u> Scudder	+			
<u>M. packardii</u> Scudder	+		+	+
<u>M. bowditchi canus</u> Hebard		+		
<u>M. confusus</u> Scudder	+			
<u>M. gladstoni</u> Scudder		+		
<u>M. femurrubrum femurrubrum</u> (DeGeer)	+	+	+	+
<u>M. infantilis</u> Scudder	+	+	+	
<u>M. differentialis</u> (Thomas)	+		+	+
<u>M. cuneatus</u> Scudder	+			
<u>M. foedus foedus</u> Scudder	+			
Miscellaneous Orthoptera				
<u>Gryllus</u> sp.		+		
<u>Tetrigidae</u> (undetermined sp.)	+			

^a Nat = natural infection; Exp = experimental infection.

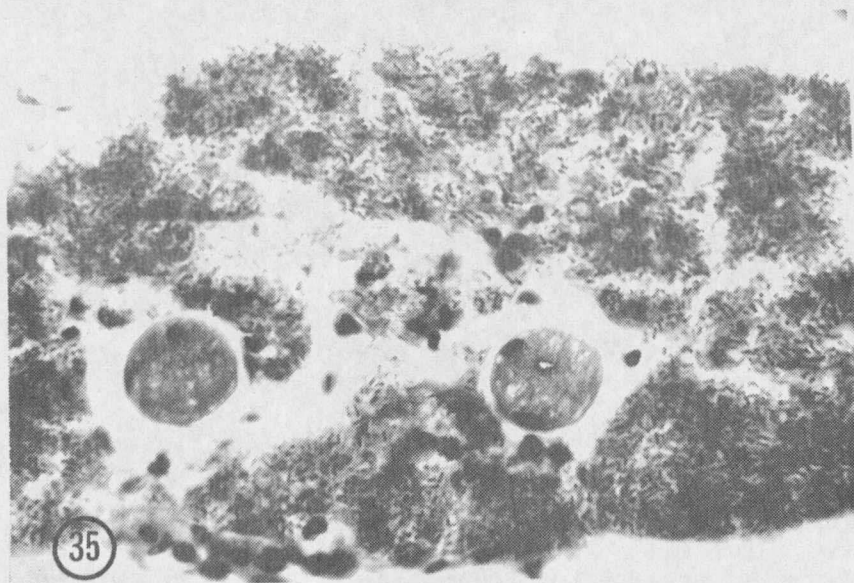
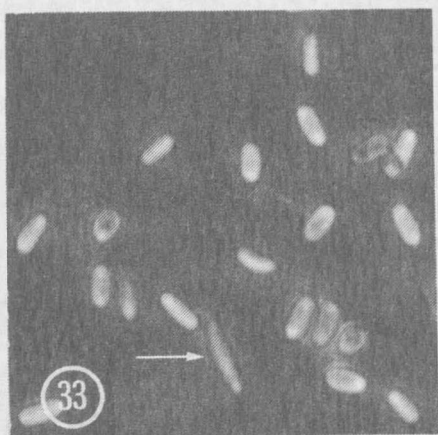
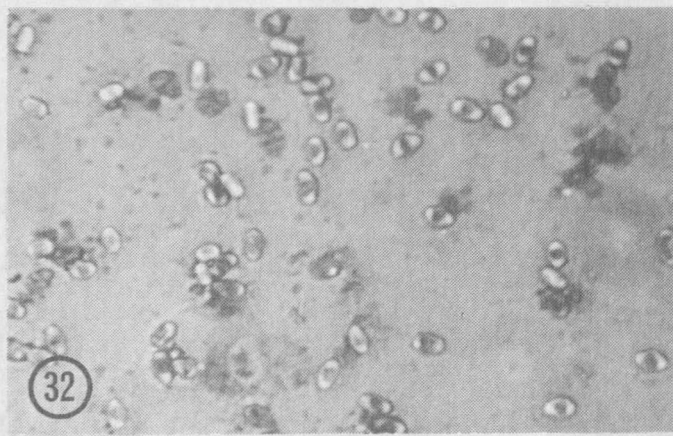
However, in two laboratory tests, the eastern house cricket, Acheta domesticus (L.) was not susceptible to N. locustae.

Stages in the Life Cycle of N. locustae

According to Canning (1962) uninucleate schizonts measured from 2.5 to 3.5 microns in diameter, binucleate schizonts measured from 3 to 5 microns, and quadrinucleate schizonts from 5 to 6.5 microns. These measurements corresponded to measurements made during the present studies of schizonts in fixed preparations with the exception that quadrinucleate schizonts measuring up to 8 microns in diameter also were observed. In fresh preparations under phase contrast microscopy, uninucleate schizonts measured up to 8 microns in diameter, binucleate schizonts from 6 to 10 microns and quadrinucleate schizonts from 7 to 13 microns.

In fixed preparations the diplokaryon forms measured about 5 microns in diameter and possessed two hemispherical nuclei which stained intensively with the Giemsa smear technique. The uninucleate sporonts also measured about 5 microns in diameter while the nucleus measured about 3 microns in diameter. The binucleate sporonts measured about 4.5 microns in diameter (Fig. 31). Each nucleus of these binucleate forms measured about 1 micron in diameter. The sporoblasts appeared rod-shaped, somewhat ellipsoidal (Fig. 32) and measured from 6.5 to 8 microns in length by about 3.5 microns in diameter. The spores of N. locustae possessed a light refractive wall, were ellipsoidal and measured 5.2 microns in length by 2.8 microns in diameter (Fig. 33). Triangulate spores and elongate megaspores, which measured about 8 microns in length, were common. The

- Fig. 31. Binucleate sporonts of Nosema locustae in a smear preparation. 2400X.
- Fig. 32. Spores and sporoblasts of Nosema locustae in a smear preparation. 1000X.
- Fig. 33. Spores, sporoblasts, and a megaspore (arrow) of Nosema locustae under phase contrast microscopy. 1400X.
- Fig. 34. Spore of Nosema locustae as observed during extrusion of the polar filament. 1400X.
- Fig. 35. Section of the fat bodies and nerve cords of a nymph of Melanoplus sanguinipes showing the heavy accumulations of spores of Nosema locustae. 340X.



polar filaments of spores of N. locustae, which were extruded by mechanical pressure (Fig. 34) and were measured while attached to spores, averaged 86 microns in length with a maximum recorded length of 145 microns.

Dissanaike and Canning (1957) studied the internal structure of the spores of N. locustae and Nosema helminthorum Moniez and reported that the polar filament was coiled within the sporoplasm and that upon extrusion the sporoplasm emerged from the spore already attached to the terminal end of the filament. Huger (1960), however, examined spores of N. locustae with the electron microscope and reported that the polar filaments were coiled around the sporoplasm and that the sporoplasm emerged from the spore after extrusion of the polar filament. Because the core of a polar filament appeared electron dense in the micrographs, he proposed that it was solid structures and that the sporoplasm diffused externally along the outstretched filament.

During the present studies spores of N. locustae usually were detected at 13 days postinoculation in experimentally infected grasshoppers reared at 30° C. under continuous illumination. Binucleate and quadrinucleate schizonts usually were evident by 6 days postinoculation.

Histopathology of Infections by N. locustae

Infections by N. locustae were observed in the fat bodies, pericardial cells and cells in the neural system. Spores were observed in the lumen of the hindgut, in feces and in the gonads, but this was considered due to the passive movements of spores into gonads during

histogenesis and through the digestive tract wall as a result of the excretory function of this organ and, possibly, the Malpighian tubules. Spores also were observed in hemolymph smears in which they appeared both free-floating and intracellular in hemocytes. Because schizonts and sporonts were not present in hemocytes, the intracellular location of these spores probably resulted from phagocytosis.

Infections by N. locustae generally were distributed throughout the fat body tissues which changed from a normal glossy white or yellow condition to flaccid white or gray color. The fat bodies of heavily infected grasshoppers were hypertrophied and consisted primarily of spores of N. locustae (Fig. 35). Moribund or dead grasshoppers frequently contained from 10^6 to 10^7 spores per milligram of grasshopper weight.

Effect of Infections by N. locustae on Grasshoppers

In Table III are shown the results of a study to determine the susceptibility of individually-reared and group-reared Melanoplus bivittatus to N. locustae. In this study the individually-reared grasshoppers exhibited high incidences of infection and high spore concentrations, but low mortality. In contrast, the group-reared grasshoppers exhibited lower incidences of infection and lower spore concentrations, but relatively high mortality. Although the lower incidence of infection and lower spore concentrations were due to the shorter postinoculation period of group-reared grasshoppers (17 days for group-reared and 25 days for individually-reared grasshoppers), the high mortality among these grasshoppers indicated that the rearing of grasshoppers in groups probably predisposed them to

TABLE III

Susceptibility of Melanoplus bivittatus to infections by Nosema locustae.

Concentration of spores	No. of grass- hoppers tested	Rate of infection		Level of infection		Percent mortality
		No. examined	Percent infected	No. counted	No. spores/mg insect wt.	
<u>Individually-reared (25 days)</u>						
5.5X10 ³	20	19	94.7	18	3.39X10 ⁶	5.0
5.5X10 ⁴	20	20	100.0	20	1.69X10 ⁷	0.0
5.5X10 ⁵	20	19	94.7	18	4.93X10 ⁷	10.0
Untreated	46	--	--	--	--	6.5
<u>Group-reared (17 days)</u>						
5.5X10 ³	54	23	34.8	8	7.75X10 ²	55.6
5.5X10 ⁴	52	25	80.0	20	3.06X10 ³	49.9
5.5X10 ⁵	61	24	100.0	24	2.29X10 ⁴	63.9
Untreated	80	--	--	--	--	38.8

an increased effect by N. locustae. The mortality among the untreated group-reared grasshoppers also was relatively high (38.8%) with the mortality among the inoculated grasshoppers only about 12 to 25% higher. It could be concluded from this that infections by N. locustae were predisposing factors which subjected the grasshoppers to increased effects from what might be considered as intra-group antagonism. Most deaths among the untreated grasshoppers resulted from cannibalism. Likewise, cannibalism apparently caused most of the deaths of the infected grasshoppers since many of the dead grasshoppers were partially consumed and the low spore concentrations in these grasshoppers showed that N. locustae had not increased to lethal concentrations during the 17-day postinoculation period. From these results, it appears that infected grasshoppers are more cannibalistic, or exhibit more intra-group antagonism, than uninfected grasshoppers.

A second study was conducted to determine the effect of N. locustae on the rate of development and mortality among individually-reared M. sanguipes. At 55 days postinoculation, when the study was terminated, the mortalities were 86.7% among the grasshoppers inoculated with 4×10^6 spores, 49.2% among those inoculated with 4×10^4 spores and 27.5% among the untreated grasshoppers. As evident in Fig. 36, the rate of mortality among the grasshoppers inoculated with 4×10^6 spores followed a stepwise pattern with increases occurring at intervals of about 13 days. Because of the 13-day developmental period of N. locustae, this stepwise pattern in the mortality rate indicated possible synchrony in the life cycle of

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the microsporidian.

In this study, the rate of development was determined by the frequency and weights of adults in the three test groups. As shown in Table IV, the infected grasshoppers developed at slower rates than the uninfected grasshoppers. For example, at 25 days postinoculation, 94.1% of the uninfected grasshoppers were adults while only about 22% of those in the treated groups were adults. The average weights of grasshoppers inoculated with 4×10^6 and 4×10^4 spores were 278 and 301 milligrams, respectively, compared to an average weight of 357 milligrams for uninfected adults.

A third study was conducted to determine the effect of N. locustae on the reproduction of M. differentialis. As shown in Table V, the grasshoppers inoculated as 4th instars produced fewer egg pods than those inoculated within 48 hours after adult emergence. A higher percentage of the grasshoppers inoculated as 4th instars was infected, indicating that the nymphs were more susceptible than adults to infection by N. locustae. Most of the grasshoppers in group 3, which were not treated and were to function as controls, became infected during the study by contamination. Nevertheless, the results of this study showed that infections by N. locustae reduced the number of egg pods deposited by grasshoppers. It was noted that many infected females laid eggs which, instead of being deposited in pods in soil, were spewed on the surface. Although not tested, the spewed eggs were considered as nonviable and were not included in the numbers of egg pods shown in the table.

TABLE IV

Effect of infections by Nosema locustae on the rate of development of Melanoplus sanguinipes as determined by the frequency of adults in the test groups.

Cumulative days post- inoculation	Percentage of grasshoppers in adult stage		
	Untreated group	Inoculated groups	
		⁴ 4X10 ⁴ spores	⁶ 4X10 ⁶ spores
15	4.6	0.0	0.0
20	75.7	2.1	9.8
25	94.1	22.2	22.1
30	98.0	45.2	40.3
35	99.0	62.8	54.9
40	99.0	70.7	59.6
45	99.0	76.7	71.4

TABLE V

Effect of infections by Nosema locustae Canning on the fecundity of Melanoplus differentialis.

Group	Developmental stage at treatment	No. spores/ grasshopper	No. grasshoppers ^{1/} examined	Percent infected	Average level of infection ^{2/}	Total egg pods/ group
1	4th instar	2X10 ⁵	27	96.2	9.31X10 ⁶	0
2	4th instar	2X10 ³	27	96.2	4.79X10 ⁶	6
3	4th instar	--	30	80.8	7.16X10 ⁶	11
4	adult	2X10 ⁵	28	51.8	9.75X10 ⁵	42
5	adult	2X10 ³	27	62.9	1.09X10 ⁶	41
6	adult	--	27	0.0	--	58

^{1/} Numbers represent the grasshoppers examined for infection. Each group was initiated with 15 males and 15 females separated in pairs.

^{2/} Based on the average number of spores per milligram of weight of infected grasshoppers only.

Transmission of *N. locustae*

Spores of *N. locustae* have been observed in fecal pellets, ovaries, and squashed preparations of eggs from infected grasshoppers. In a test of the transovarian transmission of *N. locustae*, about 5% of the nymphs hatched from eggs of three infected *M. bivittatus* females and examined as 3rd instars were infected. These observations indicated that *N. locustae* was transmitted by fecal contamination and by transovarian means, either in or on the eggs. Dead grasshoppers infected with *N. locustae* were collected from field populations both while and after being fed upon by other grasshoppers.

Nosema acridophagus Henry

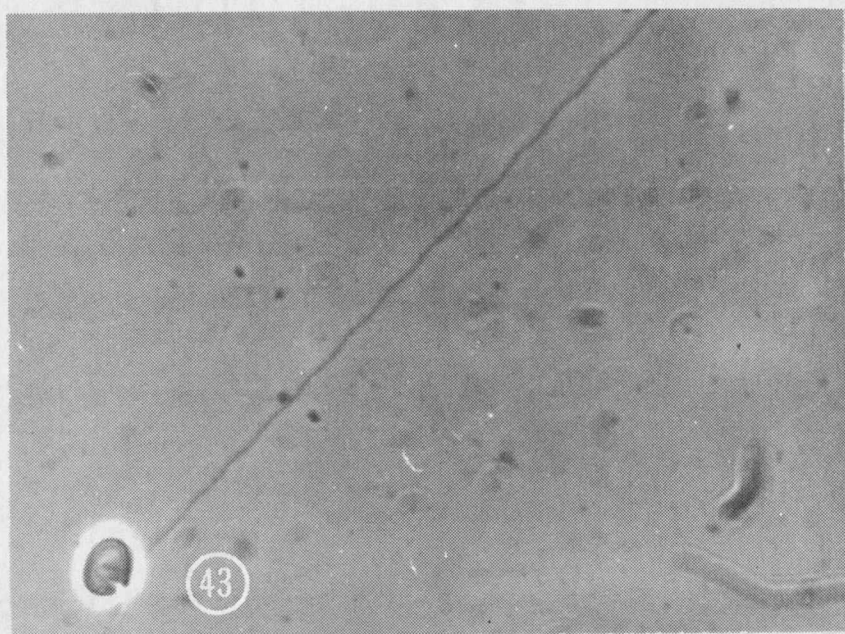
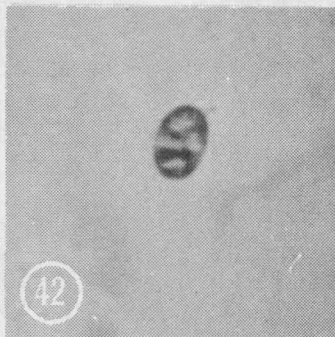
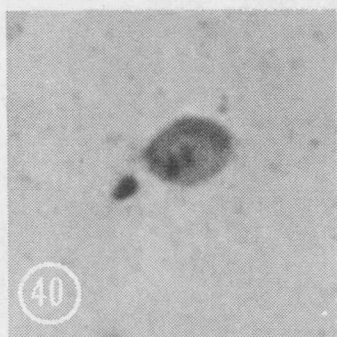
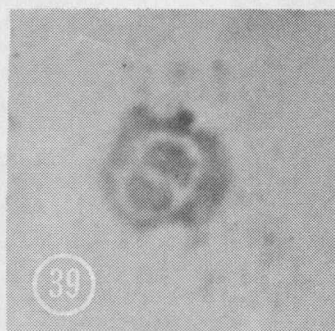
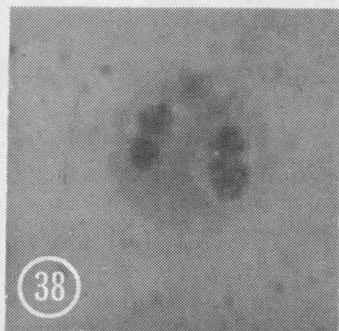
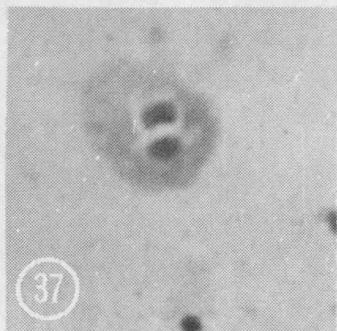
Host and Geographical Range of *N. acridophagus*

This species was isolated and described from *Schistocerca americana* which were being maintained in the laboratory following collection near Tifton, Georgia. It was transmitted experimentally to *Melanoplus sanguinipes*, *M. bivittatus*, *M. differentialis*, *S. americana*, and *Schistocerca vaga vaga*. These were the only species of grasshoppers tested for susceptibility. In two tests, the eastern house cricket, *Acheta domesticus*, was found nonsusceptible to infections by this microsporidian. *N. acridophagus* has not been isolated during these studies from grasshoppers collected in parts of western United States nor has the incidence of infections in grasshoppers in southeastern United States been determined.

Stages in the Life Cycle of *N. acridophagus*

Binucleate amoebula were observed floating free in the hemolymph or attached to the terminal ends of polar filaments which were extruded by mechanical pressure. In both fixed and fresh preparations they measured about 2 microns in diameter. Uninucleate schizonts measured from 3.0 to 5.0 microns in diameter in fixed smears and often appeared irregularly shaped. Binucleate schizonts were irregular in shape and measured 3.5 to 6.0 microns in diameter (Fig. 37). Quadrinucleate schizonts (Fig. 38) measured between 5.0 to 9.0 microns in diameter and also were irregularly shaped. All schizonts appeared spherical in fresh preparations. Diplokaryia (Fig. 39) measured about 4.5 to 6.0 microns in smears and generally appeared slightly elongate. The uninucleate sporonts generally were circular in fixed preparations and measured 4.5 to 6.0 microns in diameter. Binucleate sporonts (Fig. 40) were ellipsoidal to spherically shaped and usually measured about 4 microns in length or diameter. Spindle shaped sporonts were observed (Fig. 41) which measured about 6.0 microns in length by 3.0 microns in diameter. The sporoblasts (Fig. 42) and spores were uniformly shaped, being somewhat ovoid, and measured 4.1 microns in length by 2.6 microns in diameter in fresh preparations and 3.9 by 2.5 microns in fixed preparations. Following extrusion by mechanical pressure, the polar filaments (Fig. 43) averaged 74 microns in length with a maximum recorded length of 114 microns. Generally, spores were observed in homogenates four days after experimental infections of grasshoppers which were reared at 30° C. under continuous illumination. However,

- Fig. 37. A binucleate schizont of Nosema acridophagus. Smear preparation. 2700X.
- Fig. 38. A quadrinucleate schizont of Nosema acridophagus. Smear preparation. 2700X.
- Fig. 39. A diplokaryon form of Nosema acridophagus. Smear preparation. 2700X.
- Fig. 40. A binucleate sporont of Nosema acridophagus. Smear preparation. 2700X.
- Fig. 41. A spindle-shaped sporoblast of Nosema acridophagus. Smear preparation. 2700X.
- Fig. 42. A spore-shaped sporoblast of Nosema acridophagus. Smear preparation. 2700X.
- Fig. 43. A spore of Nosema acridophagus with an extruded polar filament. Fresh preparation under phase contrast microscopy. 2000X.



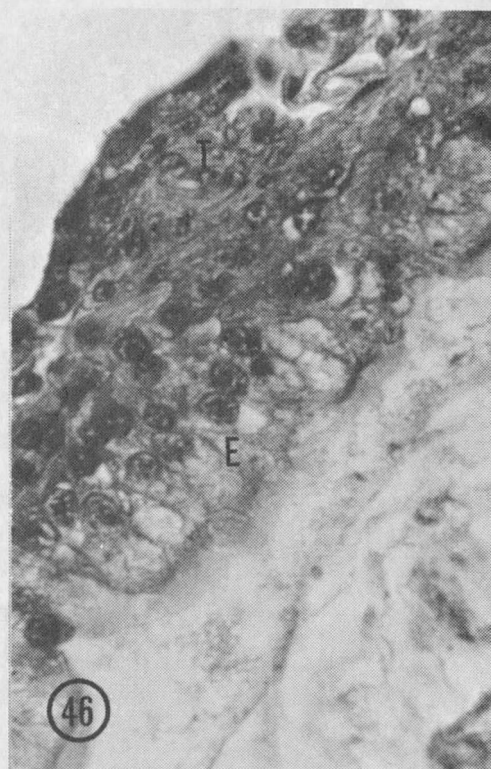
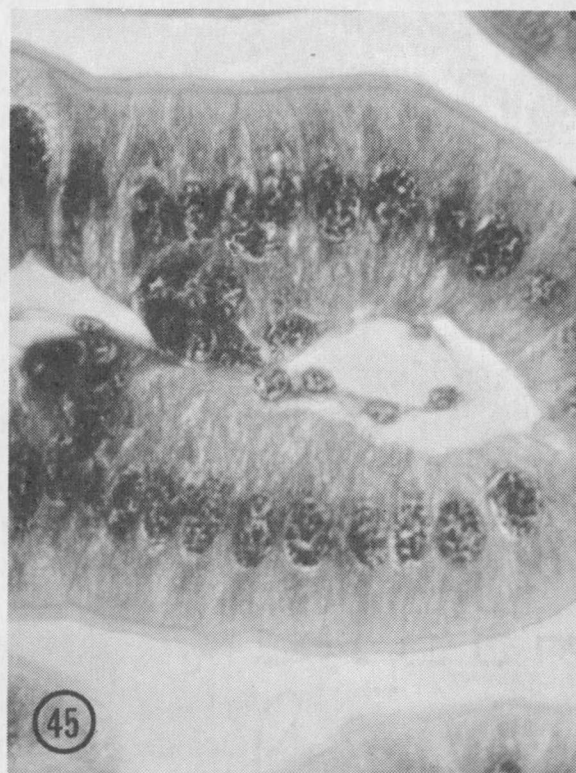
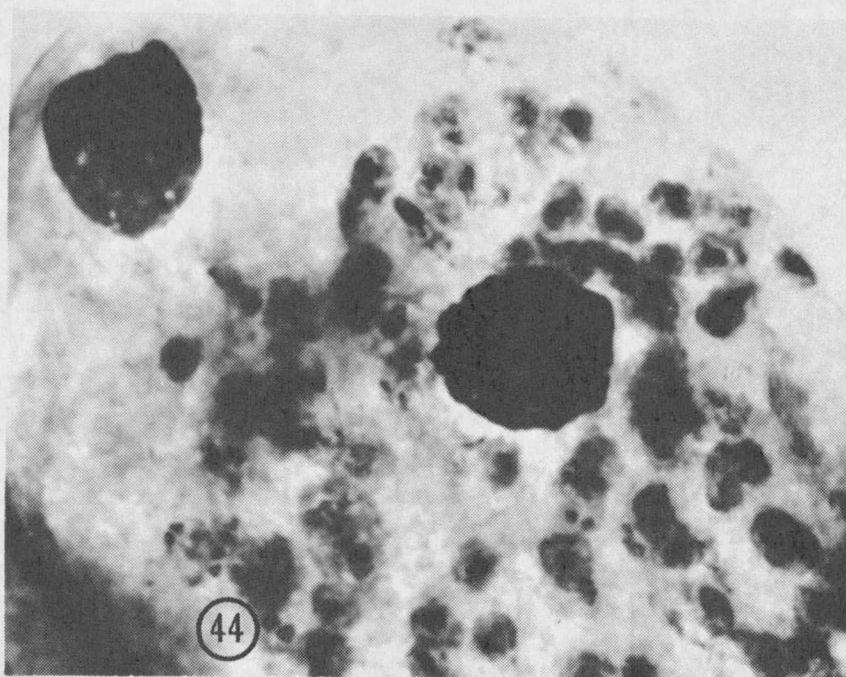
spores and sporonts also were observed in a section of midgut tissues which was prepared 48 hours after experimental infection.

Histopathology of Infections by *N. acridophagus*

Infections by *N. acridophagus* were observed in the midgut, gastric caeca, gonads, fat bodies, pericardium (Fig. 44) and neural tissues. Characteristically, *N. acridophagus* induced abnormal proliferations of cells which aggregated and formed growths and were referred to as tumors. According to Harker (1963) the term "tumor" implies cell multiplication. In gross examination of dissected grasshoppers the tumors usually appeared as small swellings of transparent tissue. The large tumors frequently included pigmented granules. Examinations of sections prepared from infected tissues showed that tumors were formed by aggregations of invading cells which disrupted the normal tissues. The formation of the tumors was considered to be the main factor responsible for the relatively high virulence associated with infections by *N. acridophagus*.

A study was made of the early morphogenesis of the tumors in which the midguts and gastric caeca of nymphs of *M. sanguinipes* were removed and fixed at 20, 48, and 72 hours after inoculations (Henry, 1969b). In the sections prepared at 20 hours postinoculation tumor development varied from the initial stages of development, as indicated by unusually high frequencies of mitotic figures in some regenerative nidi, to tumors which were several cell layers thick. The nidi which appeared to be involved in tumorigenesis were larger than those in other parts of tissue sections where there was no evidence of tumors.

- Fig. 44. Schizonts and sporonts of Nosema acridophagus in a binucleate pericardial cell. Smear preparation. 1500X.
- Fig. 45. Cross section of the convoluted epithelium of a gastric caecum from Melanoplus sanguinipes showing initial tumor formation, as indicated by the enlarged regenerative nidi, during infection by Nosema acridophagus. 500X.
- Fig. 46. Cross section of the midgut of a Melanoplus sanguinipes showing the appearance of a tumor (T) during initial formation and some vacuolization of the epithelial cells (E). 450X.



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Most tumors in the 20-hour sections were discernible as aggregations of cells in unusual locations. In the midgut these aggregations were observed along the wall of the midgut and extending into the hemocoel. In the gastric caeca they usually occurred between the folds of the convoluted epithelium (Fig. 45). Lines of cells were observed extending from nearby regenerative nidi to the site of the developing tumor. Occasionally an entire nidus appeared to have been displaced in the direction of the tumor. Bulges and lesions were observed in the basal membrane of the midgut which appeared to have been caused by the pressure exerted by proliferating and migrating cells (Fig. 46).

Except for one instance, all mitotic figures occurred in the regenerative nidi. This indicated that some factor induced the initial differentiation of nidi cells into tumorous cells prior to migration. Partial differentiation occurred during migration in that the nuclei of cells in or near the regenerative nidi appeared larger and more granular than those near or in the tumors.

The tumors in sections prepared at 48 hours postinoculation were more numerous and generally larger than those observed in the tissues prepared at 20 hours postinoculation. At 48 hours postinoculation numerous vacuoles were observed in the epithelial cells which were adjacent to the larger tumors. This vacuolated condition probably resulted from the lack of cells from regenerative nidi for replacement of spent epithelial cells. In addition, some sloughing of groups of epithelial cells was noted which caused noncellular gaps in the epithelium. Direct infection by

N. acridophagus of the epithelial or regenerative nidi cells has not been observed.

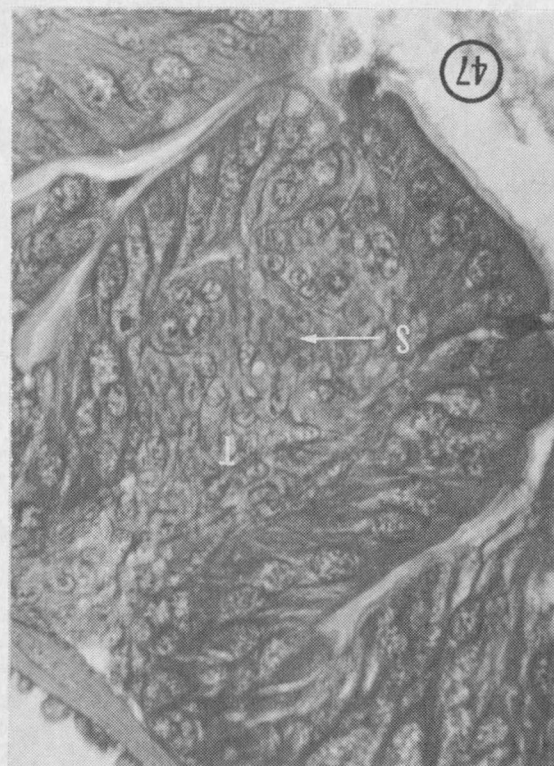
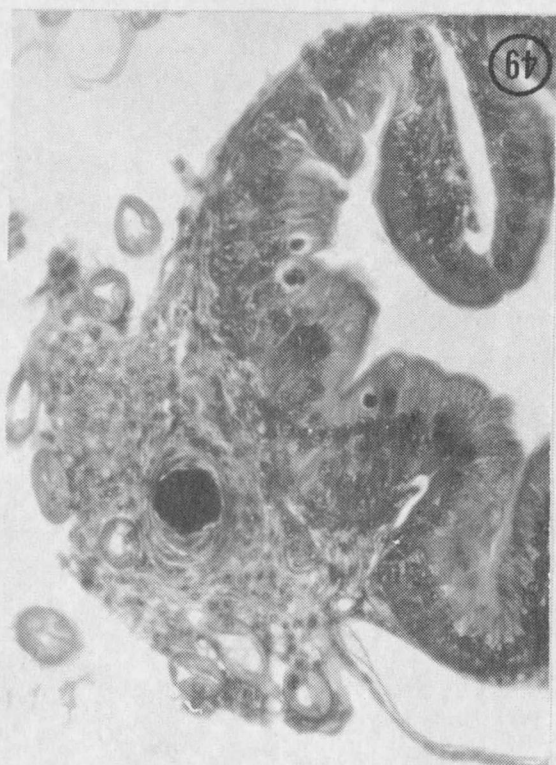
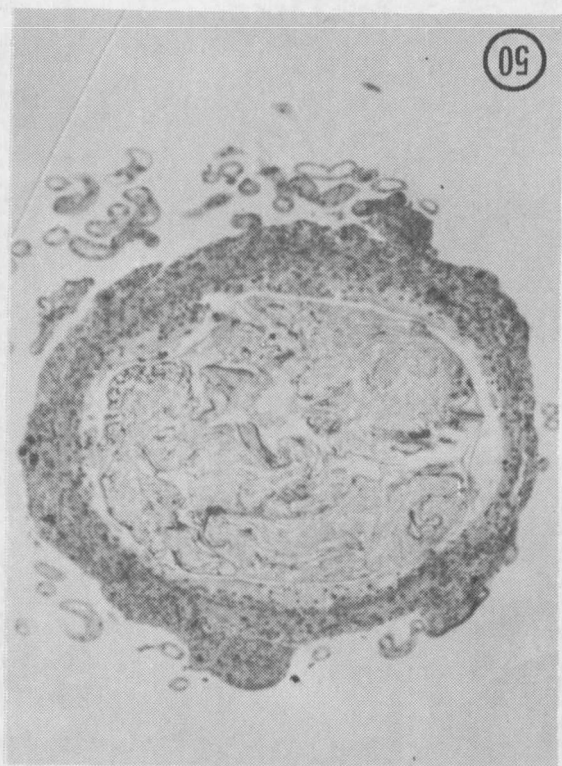
In the sections prepared 20 hours after inoculation most of the mitotic activity was confined to the nidi nearest the developing tumors while in the sections prepared 48 hours postinoculation, mitotic activity was evident in nidi which were located 200 to 400 microns away from the tumors. This indicated that the tumor-inducing factor probably diffused through the tissues. It was not determined whether these nidi produced cells which migrated to existing tumors or which formed new tumors.

As shown in Fig. 47, schizonts of N. acridophagus were observed within a 48-hour-old tumor in a gastric caecum. Also, four spores and about six sporonts were observed between the displaced layers of the basal membrane in a midgut section prepared at 48 hours postinoculation (Fig. 48). The presence of spores in this section showed that N. acridophagus was capable of completing a life cycle within 2 days and was probably capable of intercellular development. Previously Henry (1967) reported only intracellular growth.

The tumors in sections prepared at 72 hours after infection were larger, often five or more cell layers thick, and frequently contained melanotic cores. The larger tumors also exhibited a characteristic swirling appearance (Fig. 49). Extensive degradation of the epithelium was observed in the vicinity of the larger tumors.

Other sections of midguts and gastric caeca from M. sanguinipes and S. vaga vaga were prepared from 5 to 10 days after inoculation. The tumors

- Fig. 47. Cross section of a gastric caecum of a Melanoplus sanguinipes which was prepared at 48 hours after inoculation with Nosema acridophagus showing a tumor (T) which encompasses schizonts (S) of the microsporidian. 400X.
- Fig. 48. Cross section of the midgut of a Melanoplus sanguinipes showing a tumor (T) and spores and sporonts of Nosema acridophagus (arrows) between layers of the basal membrane. 1500X.
- Fig. 49. Cross section of a gastric caecum of a Melanoplus sanguinipes prepared at 72 hours after inoculation with Nosema acridophagus showing a large tumor with a melanotic core. 200X.
- Fig. 50. Cross section of the posterior region of the midgut of a Schistocerca americana showing tumors induced by Nosema acridophagus encircling the digestive tract. 80X.



in these sections were extensive and occasionally encircled the posterior region of the midgut (Fig. 50). In such cases the basal membrane of the midgut appeared totally disrupted and lesions were evident. The epithelium was either lacking or degraded to a layer of irregularly arranged cells. Melanotic structures were common throughout the tumor and spores of N. acridophagus usually were evident within the melanized structures.

Apparently most tumors in the midgut and gastric caeca were formed by cells from the regenerative nidi. However, in some sections prepared at or after 72 hours postinoculation, some unattached cells were observed near the surface of the larger tumors which protruded into the hemocoel. This indicated that cells from the hemocoel might take part in forming tumors. However, it was also possible that these cells were torn loose from the tumor during sectioning. Although the origin of tumors in tissues in the hemocoel was not investigated, it appeared unlikely that the regenerative nidi were involved. According to Matz (1965) both blood cells and nidi cells formed tumors in locusts following severance of the sympathetic nerves. He also reported that the experimentally induced tumors were transmissible by means of acellular extracts. Although attempts were not made to transmit the tumors induced by N. acridophagus, the fact that the tumor-inducing factor spreads through tissues indicates that such transmission might be possible.

Gross Effects of Infections by N. acridophagus

N. acridophagus was considered as a moderately virulent pathogen in

that, following inoculation with 10^5 spores, 3rd instar nymphs exhibited gross symptoms within 6 days and 50% mortality within 10 days. Moribund grasshoppers lacked normal coordination, were unable to remain standing and exhibited uncontrolled leg movements. Bacterial septicemia frequently was evident from smears of the hemolymph of moribund grasshoppers.

The data from a study of the susceptibility of individually-reared and group-reared M. bivittatus to infections by N. acridophagus are shown in Table VI. In comparison with the results of a similar study conducted with N. locustae (Table III), N. acridophagus caused higher mortality, higher rates of infection, but fewer spores were produced. Unlike N. locustae, however, there was less difference in the response of group-reared and individually-reared grasshoppers to N. acridophagus. Apparently this was due to the shorter time necessary for N. acridophagus to complete a life cycle. In effect, this pathogen proliferated more rapidly and reached lethal concentrations within the 17-day postinoculation period of the group-reared grasshoppers.

Melanoplus sanguinipes, M. bivittatus, M. differentialis and Schistocerca vaga vaga were used for studies of N. acridophagus. Observations indicated little or no differences in the susceptibility of these species to this pathogen. However, M. bivittatus responded differently to infections in that the tumors in this species were smaller, more abundant and more widely scattered. Usually most tumors in the hemocoel of the other three species were located in the vicinity of the midgut, while in M. bivittatus they appeared evenly distributed throughout the hemocoel.

TABLE VI

Susceptibility of Melanoplus bivittatus to infections by Nosema acridophagus.

Concentration of spores	No. of grass- hoppers tested	<u>Incidence of infection</u>		<u>Level of infection</u>		Percent mortality
		No. examined	Percent infected	No. counted	No. spores/mg insect wt.	
<u>Individually-reared (25 days)</u>						
5.5X10 ³	20	19	94.7	18	1.21X10 ⁵	85.0
5.5X10 ⁴	20	20	100.0	20	1.19X10 ⁵	100.0
5.5X10 ⁵	20	19	100.0	19	1.30X10 ⁵	100.0
Untreated	46	--	--	--	--	6.5
<u>Group-reared (17 days)</u>						
5.5X10 ³	52	32	90.4	29	1.68X10 ⁴	46.2
5.5X10 ⁴	54	20	100.0	20	9.5X10 ⁴	91.7
5.5X10 ⁵	54	33	100.0	33	3.08X10 ⁴	98.1
Untreated	80	--	--	--	--	38.8

It was observed that 3rd instar nymphs were more susceptible to infections than were 5th instar nymphs and adults.

Transmission of *N. acridophagus*

Spores of *N. acridophagus* were found in the feces eliminated by infected grasshoppers. Because of this, transmission on contaminated food materials seemed likely. Also, because of infections of the gonads, transovarian transmission seems possible. However, spores of *N. acridophagus* have not been observed in squash preparations of eggs from infected females. Partially consumed cadavers of infected grasshoppers were observed which indicated that transmission by cannibalism was highly probable.

A *Nosema* sp.

Host and Geographical Range of the *Nosema* sp.

This species was isolated from *Melanoplus confusus* which were collected from the margins of an alfalfa field about 20 miles north of Bozeman, Montana. During several subsequent seasons it was isolated from approximately 20 *M. sanguinipes* and *M. bivittatus* which were collected in the same area. In the laboratory, the *Nosema* sp. was transmitted to *Melanoplus sanguinipes*, *M. bivittatus*, *M. femurrubrum*, *M. differentialis*, *M. infantilis*, and *Schistocerca americana*.

Stages in the Life Cycle of the *Nosema* sp.

The schizogony and sporogony stages of the *Nosema* sp. were similar to

the stages observed in the life cycles of N. locustae and N. acridophagus. The amoebula of the Nosema sp. measured about 3 microns in diameter in fresh preparations. The nuclei of amoebula were not observed. Uninucleate schizonts were spherical and measured about 4 microns in diameter in fresh preparations. In smears they were irregularly shaped and measured about 3 microns in diameter. Binucleate schizonts measured from 3 to 6 microns in smears and 5 to 8 microns in fresh preparations. Terminal schizonts, which were quadrinucleate, measured between 5 to 7 microns in smears and between 7 to 11 microns in fresh preparations.

The stages of sporogony were indistinguishable from those of N. acridophagus except that spindle-shaped sporoblasts were not observed in infections by the Nosema sp. All sporoblasts of the Nosema sp. appeared ellipsoidal to slightly oval and measured about 5.5 microns in length by 3.5 microns in diameter. Some unusually large sporoblasts were observed which measured about 8 microns in length by 6.5 microns in diameter.

The spores of the Nosema sp. were light refractive and oval shaped (Fig. 51). Based on the measurements of spores from a M. sanguinipes, the typical spores averaged 4.8 microns in length by 3.4 microns in diameter. Atypically large spores (megaspores) were common and measured 6.5 microns in length by 5.0 microns in diameter. Generally, the megaspores made up less than 1% of the sample; however, spore samples consisting of about 5% megaspores have been observed which indicated that the frequency of megaspores varied between individual grasshoppers. The polar filaments of typical spores (Fig. 51) measured up to 110 microns in

length while those of megaspores were longer and appeared heavier. The polar filament of the megaspore in Fig. 52 measured about 225 microns in length. Spores of the Nosema sp. usually were observed in grasshoppers within 9 days after experimental infection.

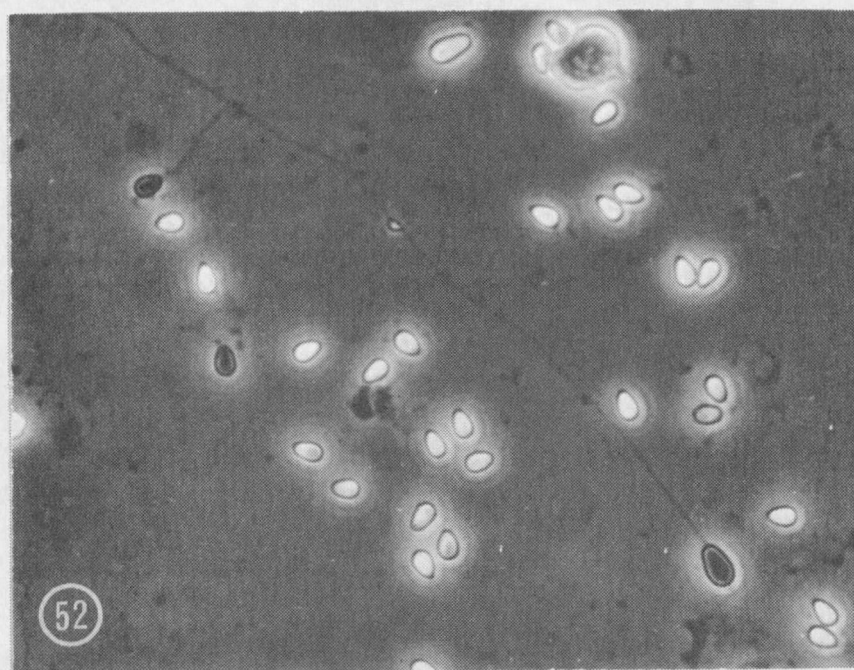
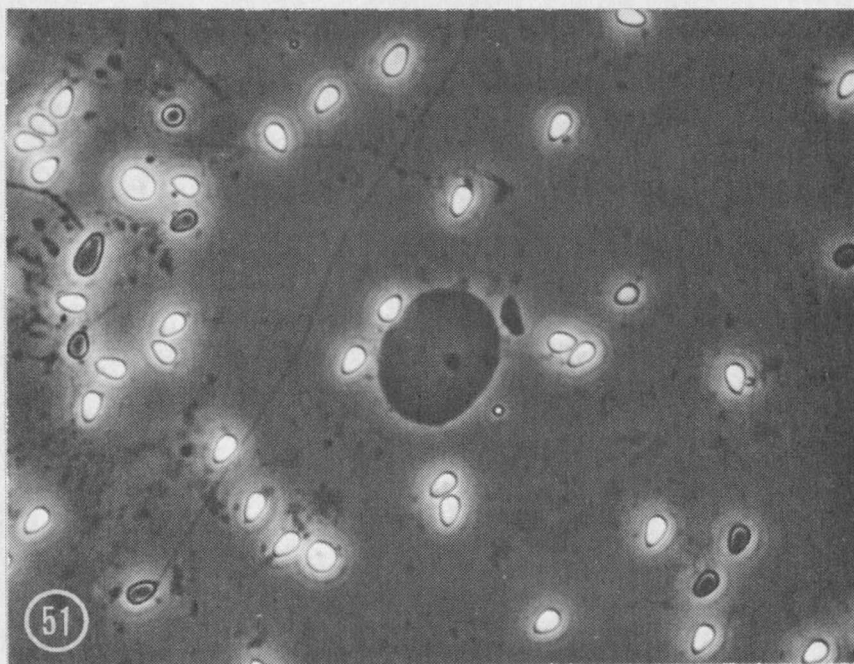
Histopathology of Infections by the Nosema sp.

Intracellular infections by the Nosema sp. were observed in cells of the fat bodies, pericardium, gonads, tracheal matrix, and midgut epithelium. Spores also were observed in the lumen of Malpighian tubules and in neural tissues where they occurred between fibers in the nerve cords and in ganglia.

Initial infections usually were restricted to one or several cells in different parts of grasshoppers. Often, in examinations of pericardial tissues, one or two cells in a microscopic field were heavily infected while all other cells appeared normal. More advanced infections also were restricted to particular sites, but most of the cells at these sites were infected and extracellular spores were observed. The infection sites were visible under a dissection microscope and appeared as small transparent nodules or swellings. In terminally infected grasshoppers the infection sites appeared as flaccid white growths which, when examined microscopically, consisted of spores of the Nosema sp. Generally only two or three such masses were present in terminally infected grasshoppers. However, some spores also were present in most other tissues of the moribund or dead grasshoppers.

Fig. 51. Fresh preparation under phase contrast microscopy of the spores of Nosema sp. showing an extruded polar filament from a typical spore. 1000X.

Fig. 52. Fresh preparation under phase contrast microscopy showing spores of Nosema sp. and an extruded polar filament from a megaspore. 1000X.



Gross Effects of Infections by the *Nosema* sp.

Gross symptoms such as general torpor, inactivity and reduced feeding activity were evident within 10 days after infection. Moribund grasshoppers were totally inactivated and exhibited tetanic convulsions.

In Table VII are shown data derived from a study similar to studies with *N. locustae* and *N. acridophagus* on the susceptibility of individually-reared and group-reared *M. bivittatus* to the *Nosema* sp. As shown, the percent mortalities among all groups, except the individually-reared grasshoppers inoculated with 5500 spores, were higher than the mortality which occurred among the respective untreated control groups. The percentage of infected grasshoppers in the different test groups corresponded directly with the mortalities. These data show that the *Nosema* sp. was sufficiently virulent to cause increased mortalities. In addition, mortalities among the group-reared grasshoppers were considerably higher than either the group-reared untreated grasshoppers or the individually-reared infected grasshoppers. The relative levels of these mortalities suggested that the group-rearing condition may have predisposed the grasshoppers to increased effects from infection. Based on spore counts in dead grasshoppers, the lethal spore level for grasshoppers reared individually was estimated to be about 1.2×10^6 spores while for grasshoppers reared in groups the lethal concentration was estimated at about 4.0×10^5 spores. These counts also showed that on an individual spore basis, the virulence of the *Nosema* sp. was intermediate between that of *N. acridophagus* and *N. locustae*.

TABLE VII

Susceptibility of Melanoplus bivittatus to infections by the Nosema sp.

Concentration of spores	No. of grass- hoppers tested	<u>Incidence of infection</u>		<u>Level of infection</u>		Percent mortality
		No. examined	Percent infected	No. counted	No. spores/mg insect wt.	
<u>Individually-reared (25 days)</u>						
5.5X10 ³	20	20	15.0	3	1.18X10 ⁶	5.0
5.5X10 ⁴	20	19	73.7	14	2.95X10 ⁵	40.0
5.5X10 ⁵	20	18	88.9	16	2.94X10 ⁵	75.0
Untreated	46	--	--	--	--	6.5
<u>Group-reared (17 days)</u>						
5.5X10 ³	55	27	18.5	5	1.48X10 ⁴	50.1
5.5X10 ⁴	53	22	63.6	14	6.43X10 ⁴	77.4
5.5X10 ⁵	53	16	81.2	13	2.38X10 ⁵	90.6
Untreated	80	--	--	--	--	38.8

Most laboratory studies of the Nosema sp. were conducted through infection of M. sanguinipes, M. bivittatus and S. americana. In these studies the Melanoplus spp. appeared more susceptible than S. americana to the Nosema sp. Third instar nymphs were more susceptible or easier to infect, than were late instar nymphs or adults.

Transmission of the Nosema sp.

Spores of the Nosema sp. were present in the fecal material eliminated by infected grasshoppers and, therefore, transmission on contaminated food appeared likely. Because of the infection of so many different tissues, transovarian transmission and transmission by cannibalism also appeared probable.

Comparative Susceptibility of Melanoplus bivittatus to Nosema locustae, Nosema acridophagus, and the Nosema sp.

The data expressed in Tables III, VI, AND VII on the susceptibility of M. bivittatus to N. locustae, N. acridophagus, and the Nosema sp. were derived from separate tests in a single study. Comparison of the results shows that M. bivittatus was most susceptible to infections and the effects of infection by N. acridophagus. The grasshoppers which were inoculated with this organism generally showed higher percentages of infection and higher percent mortality. M. bivittatus also appeared very susceptible to infections by N. locustae, as shown by the relatively high percentages of infection, but because of the low virulence associated with infections by this species, there was very little effect on the survival of the

grasshoppers. The Nosema sp. was intermediate between N. locustae and N. acridophagus in the percentages of infection, percent mortality, and levels of infection.

In Figures 53 and 54 are shown the mortality rates caused by each of the Nosema spp. during the postinoculation periods of individually-reared and group-reared grasshoppers. Besides the large difference in the total mortalities caused by the pathogens among the individually-reared grasshoppers (Fig. 53) there were differences also in the time at which these mortalities occurred. For example, N. acridophagus caused increased rates of mortality at about 10 days postinoculation and the Nosema sp. caused increased mortality rates at 18 days postinoculation. In each case, the increased mortalities occurred at about twice the time required for a life cycle of these species. Apparently, because of the shorter life cycle, N. acridophagus increased to lethal levels more rapidly than either of the other two species. The Nosema sp. probably was more virulent than N. locustae because of a shorter developmental period.

The rates of mortality associated with these microsporidians among the group-reared grasshoppers were similar during the first 10 days postinoculation (Fig. 54). During this 10-day period the mortality rates among the infected grasshoppers were from 12 to 20 percent higher than those of the untreated grasshoppers. At 10 days postinoculation there was an increase in the rate of mortality among the grasshoppers inoculated with N. acridophagus. This similarity in the mortality rates during the first 10 days postinoculation showed that the relative virulence of each

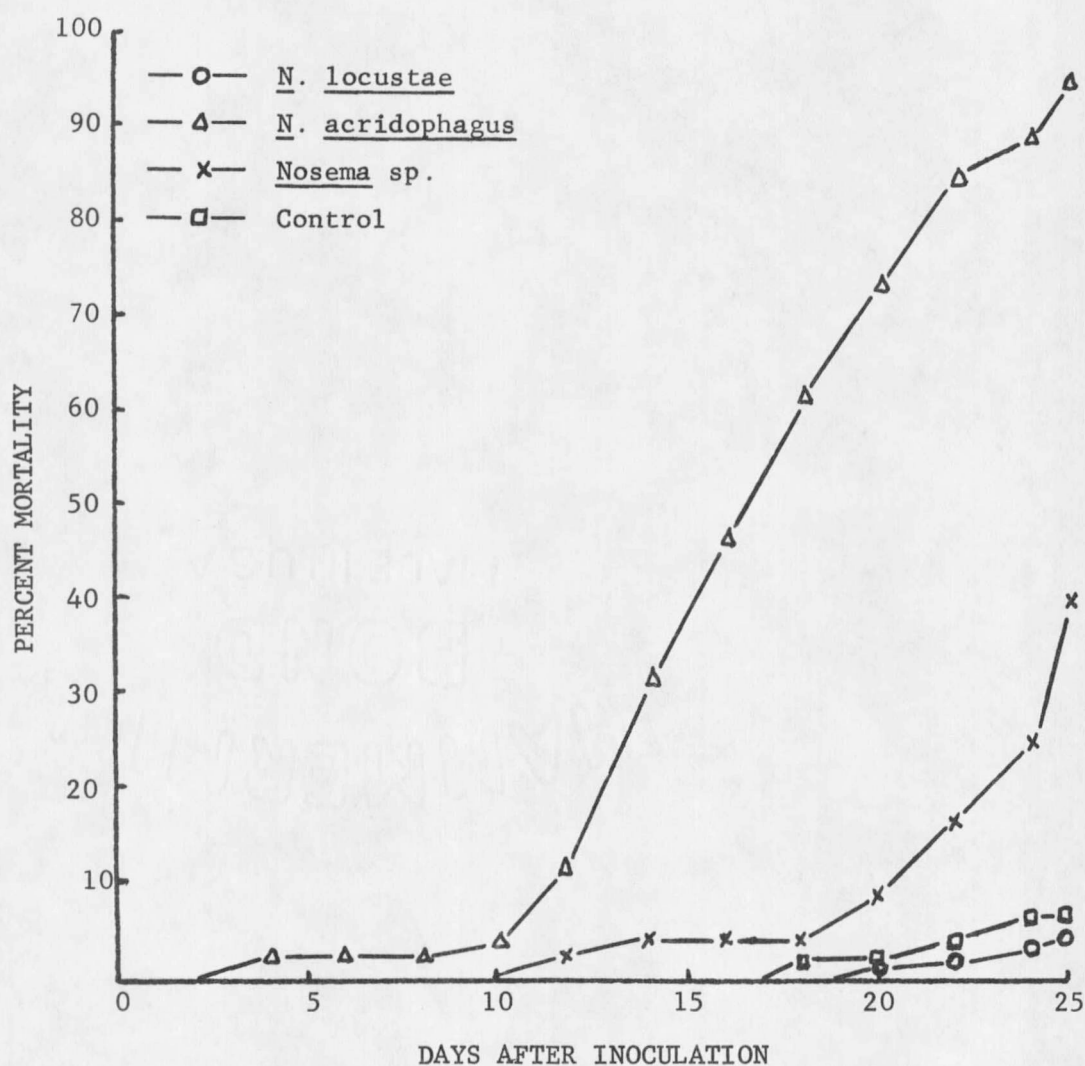


Fig. 53.--Rates of mortality among individually-reared Melanoplus bivittatus following inoculation with Nosema locustae, Nosema acridophagus, or Nosema sp.

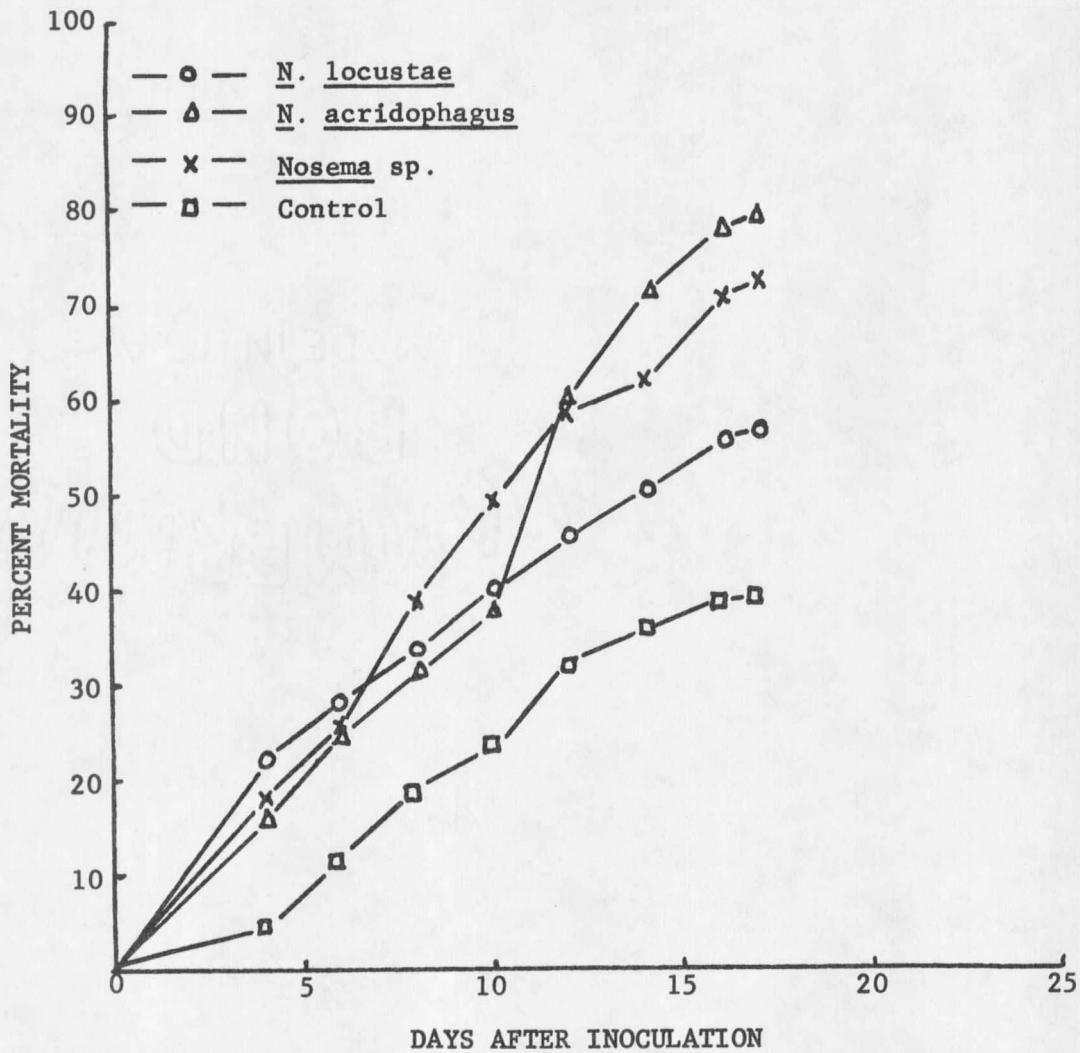


Fig. 54.--Rates of mortality among group-reared *Melanoplus bivittatus* following inoculation with *Nosema locustae*, *Nosema acridophagus*, or *Nosema* sp.

pathogen was of less importance in the presence of a second factor, which in this case was the frequency of cannibalism among the group-reared grasshoppers. Since none of the pathogens had reached lethal concentrations in the first 10 days postinoculation, as shown by the mortality rates among individually-reared grasshoppers, the infections apparently acted as predisposing factors for increased mortalities due to cannibalism.

Small Spore Diseases of Grasshoppers

Frequently during these studies small spores were observed in homogenates of field-collected grasshoppers. The spores were light refractive, ellipsoidal to rod-shaped, and usually measured about 2.5 microns in length by 1 micron in diameter. Occasional elongate forms which measured up to 4 microns in length also were observed. Attempts to transmit these spores to laboratory-reared grasshoppers were unsuccessful.

Grasshoppers containing small spores were collected in Montana, North Dakota, Idaho, Wyoming, Oregon, and Arizona. Generally the spores were more common in one or several of the species of grasshoppers from a particular area. For example, they occurred only in the slant-faced grasshoppers, particularly Chorthippus curtipennis, in collections containing many species of grasshoppers from northwestern Montana. During the 6-year study of grasshoppers of Camas County, Idaho, in which over 30,000 grasshoppers representing about 50 different species

were examined, the small spores occurred mainly in Melanoplus sanguinipes. Generally, the spores occurred in concentrations of 10^5 spores or less per milligram of grasshopper weight; however, concentrations between 10^6 to 10^7 spores also were observed.

Examinations under phase contrast microscopy showed that spores occurred intracellularly in one or a few tissues, particularly the pericardium, fat bodies, gonads, Malpighian tubules, midgut, and gastric caeca epithelium. There appeared to be differences in tissue specificities in relation to the species of host and the collection site.

Because of these differences in host species and tissue specificities, along with some minor differences in spore morphology, it appeared probable that these small spores represented an unknown number of similar species of organisms. In view of the inability to transmit these organisms, it was not possible to determine how many species were represented. Originally they were considered as probable spores of fungi; however, vegetative stages of fungi have never been observed, nor did these organisms grow on a number of fungi media, including media supplemented with grasshopper homogenates.

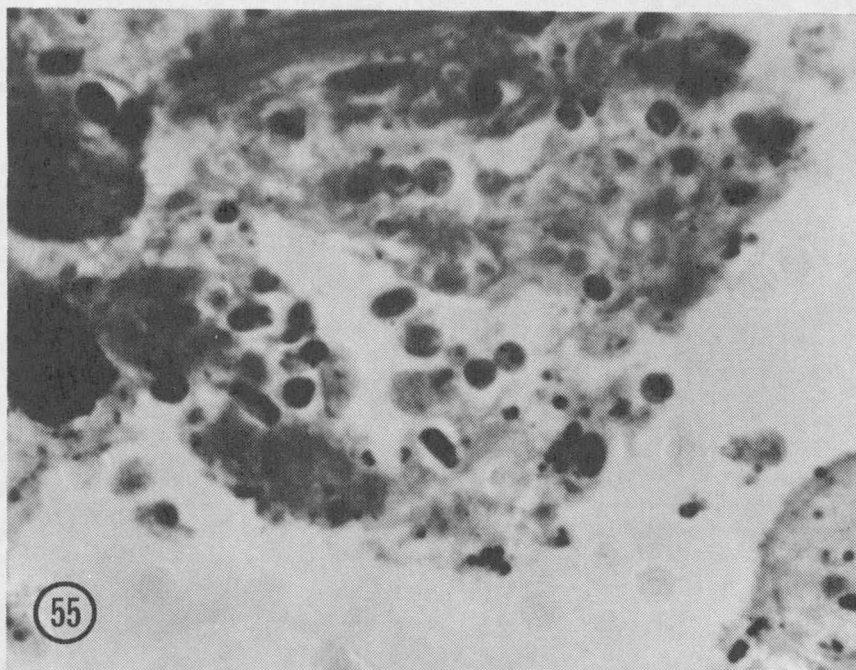
Although the possibility that at least some of the small spores might represent obligate fungal pathogens has not been ruled out, the most recent observations indicated that they probably belonged to the order Microsporida. Structures resembling schizonts and sporonts were observed along with spores in homogenates of some of the grasshoppers. Also, small spores, uninucleate sporonts, and uninucleate, binucleate,

Fig. 55. Section of the fat bodies of a Hadrotettix trifasciatus showing spores, sporonts and schizonts of a small spore type organism. 2000X.

GILBERT

Supernase

25% COTTON



55

and quadrinucleate schizonts, measuring up to 4 microns in diameter, were observed in sections of fat bodies from Hadrotettix trifasciatus from Arizona (Fig. 55). The fat bodies from the H. trifasciatus also appeared tumorous and the cell nuclei contained numerous rod-shaped granules. In electron micrographs, the granules resembled the granulosis viruses of insects. Efforts to extrude polar filaments from the spores were not successful.

The Grasshopper Inclusion Body Virus (GIBV)

Host and Geographical Range of the GIBV

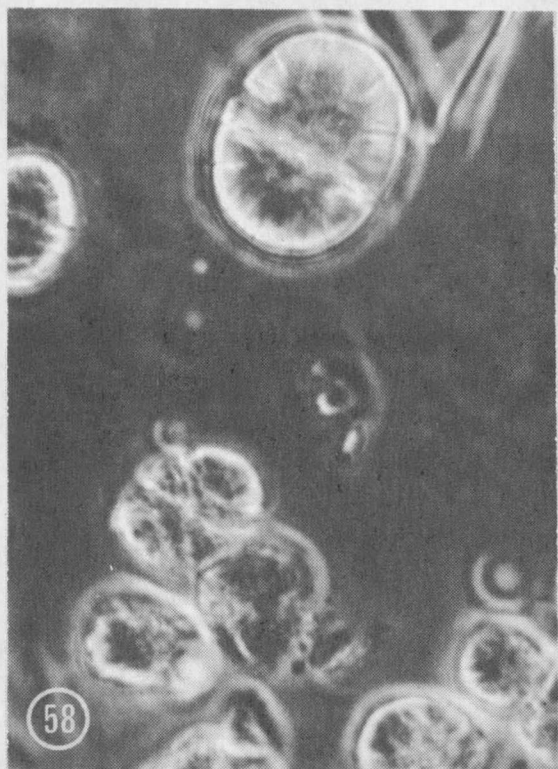
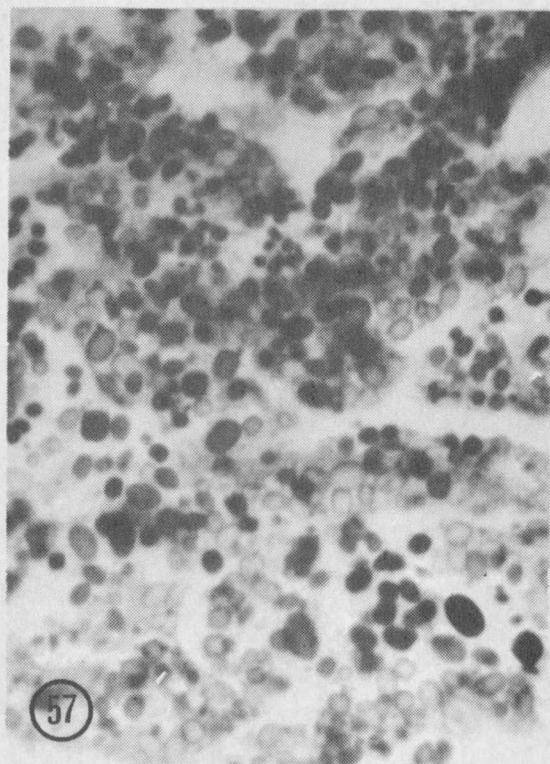
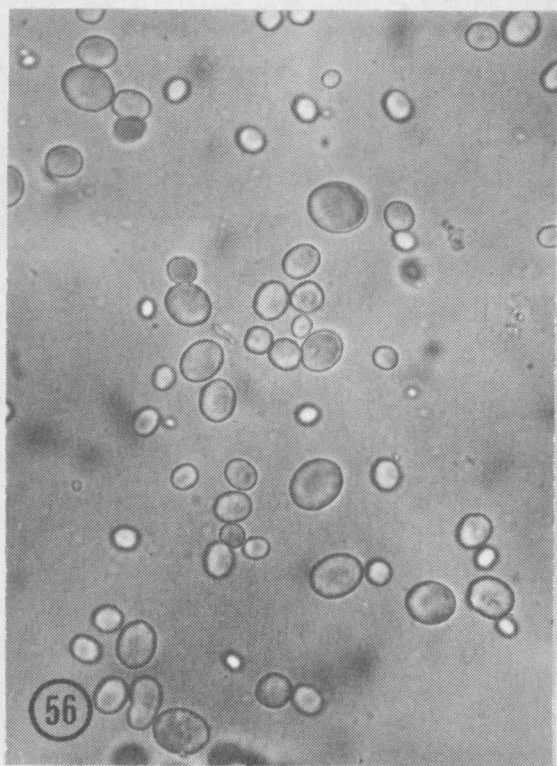
This virus was originally isolated from an adult female of Melanoplus sanguinipes in a collection of several thousand grasshoppers from the San Carlos Indian Reservation in Arizona. Later it was isolated from Melanoplus sanguinipes, M. oregonensis, M. bruneri, M. borealis, M. bivittatus, M. alpinus, and Camnula pellucida in Montana, and from M. sanguinipes from Idaho. The GIBV was diagnosed in M. sanguinipes, M. infantilis, Hypochlora alba, Phoetaliotes nebrascensis, and C. pellucida collected on study plots which had been experimentally treated with materials containing this virus. In the laboratory, the virus was transmitted to M. sanguinipes, M. bivittatus, M. differentialis, M. femurrubrum and Schistocerca americana.

Morphological and Chemical Characterization of the GIBV

Most inclusion bodies appeared ellipsoidal when observed along their longest axis and square when observed along their shortest axis (Fig. 56 and 57). They were light refractive and measured up to 10.5 microns in

- Fig. 56. Inclusion of the GIBV in a fresh preparation under bright light microscopy. 800X.
- Fig. 57. Inclusions of the GIBV in a sectioned preparation of the fat bodies of a Melanoplus sanguinipes. 650X.
- Fig. 58. Fresh preparation under phase contrast microscopy of inclusions of the GIBV during initial degradation in a 0.1% solution of sodium hydroxide. 2600X.
- Fig. 59. Fresh preparation under phase contrast microscopy of the expanded inclusions of the GIBV containing the viral particles following treatment with a 0.1% solution of sodium hydroxide. 2000X.

5% COTTON



10

5% COTTON

length but usually averaged about 5.5 microns in length by 3.7 microns in diameter. Irregularly shaped inclusion bodies were common. Inclusions generally were separate bodies, but occasionally aggregated units consisting of from two to five fused inclusions were observed. The inclusions were insoluble in water, but dissolved in weak solutions of potassium hydroxide and sodium hydroxide. During degradation in hydroxide solutions the inclusions fractured at first (Fig. 58) and then expanded to about three times their normal size and changed from the normal light refractive state to a transparent state (Fig. 59). Numerous small granules were observed within the expanded inclusion. Eventually both the inclusions and granules dissolved. The rate at which this process occurred depended on the strength of the hydroxide solutions. In 0.1% sodium or potassium hydroxides the inclusions and particles dissolved in about 30 minutes, but in 1.0% solutions they dissolved in less than 5 minutes.

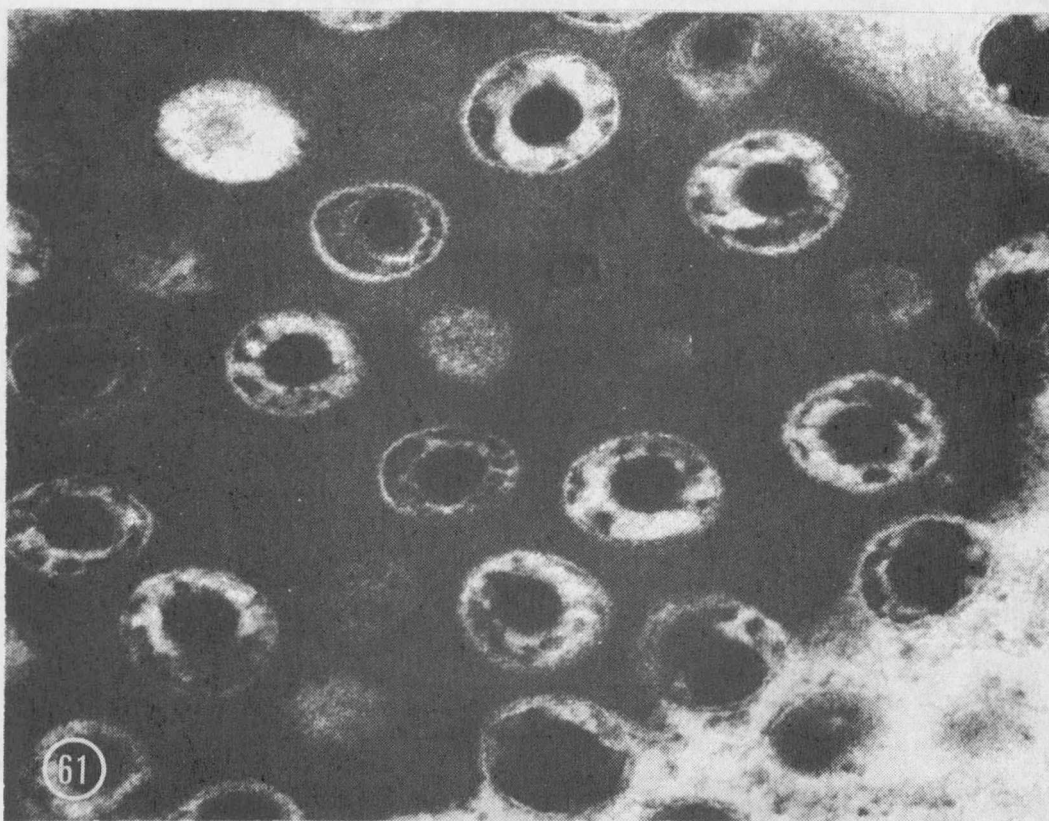
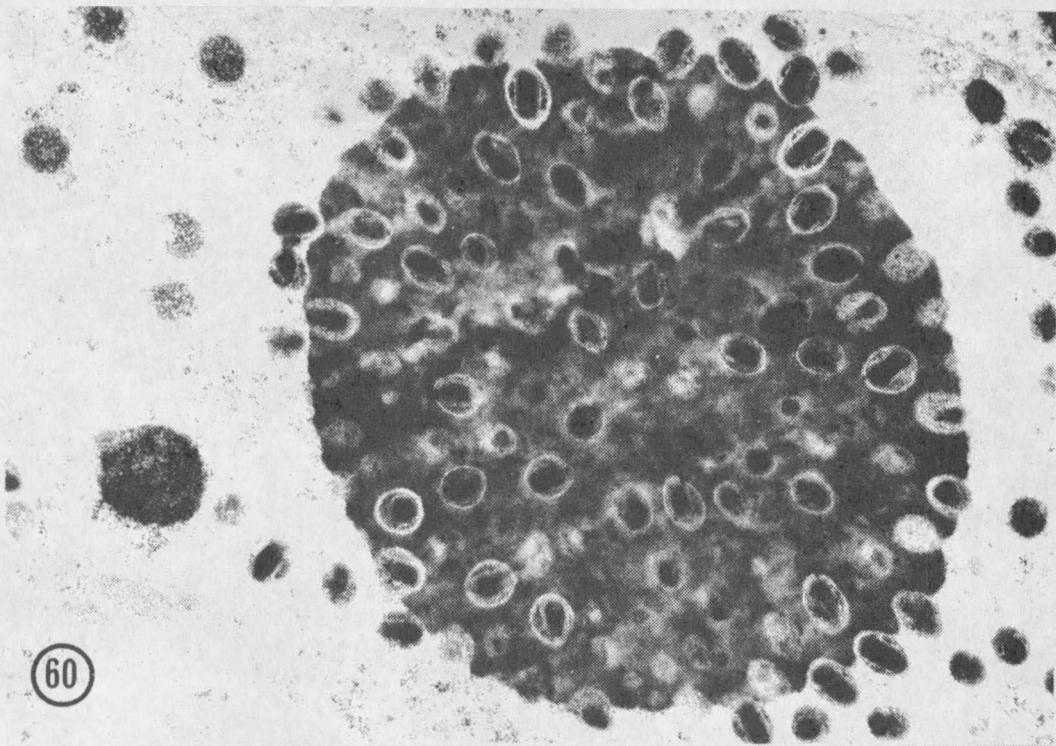
Electron microscopic examinations of ultra-thin sections of the inclusion bodies revealed rod-shaped viral particles randomly interspersed within the finely granular or crystalline matrix of the inclusion body (Fig. 60). The particles measured about 300-340 millimicrons (m μ) in length by 250-260 m μ in diameter. Each particle consisted of an electron dense central core surrounded by two membrane-like layers (Fig. 61). It was estimated that the larger inclusions contained in excess of 1000 virus particles.

Purified concentrations of inclusion bodies and free (nonoccluded)

Fig. 60. Ultra-thin section of an inclusion of the GIBV during development showing the regular dispersal of viral particles. 23,200X.

Fig. 61. Ultra-thin section of a developing inclusion of the GIBV showing the structure of the viral particles. 59,400X.

25% CO₂ CON



hyperactive

particles were obtained by density gradient ultracentrifugation of viral-rich homogenates from infected grasshoppers. About 1.2 ml of the homogenates were layered on the top of gradients consisting of 50%, 60%, 70%, 80% sucrose in water and centrifuged at 35,000 rpm for 3 hours at 2° C. in a Beckman Model L ultracentrifuge with a SW-39 head. Three light refractive zones were observed; a top zone located at 0.5 to 0.7 cm below the meniscus, a middle somewhat brownish zone at 2.2 to 2.4 cm below and a bottom zone at 2.6 to 2.8 cm below the meniscus. The zones were collected separately by a bottom puncturing technique using a 26-gauge hypodermic needle. Microscopic examinations of the zone samples revealed the presence of numerous small particles, presumed to be non-occluded viruses, in the top zone, cellular debris and inclusions in the middle zone and inclusions in the bottom zone. Recentrifugation of the middle zone sample in sucrose gradients resulted in the reappearance of the middle zone, although more widely dispersed, and a bottom zone. Apparently some inclusions from the first run middle zone were released and accumulated in the bottom zone of the second run. The viral nature of the samples of the three zones was confirmed by bioassay in laboratory grasshoppers by use of both injection and per os inoculation techniques.

Spectrophotometric assays of the fractions were carried out at wave lengths of 280 and 260 mu using a Beckman DB spectrophotometer. Analysis of the top zone resulted in a 280/260 U.V. absorption ratio of between 0.7 and 0.8 which according to Layne (1957) indicated a 6 to 10% nucleic

acid content. The 280/260 ratio for the bottom zone varied from 1.35 to 1.60 which indicated a 0.25 to 1.0% nucleic acid content. Using the method of Schneider (1957) the nonoccluded viruses (top zone) consisted of about 5% DNA while the inclusions (bottom zone) consisted of 0.9 to 1.0% DNA. No RNA was detected in the top zone fractions, but trace amounts were detected in the inclusions.

Generally these results were consistent with the nucleic acid content of the nuclear polyhedrosis viruses of other insects. According to Bergold (1963), the function of the RNA in the inclusions of the nuclear polyhedrosis viruses has not been established. However, after observing electron micrographs showing partially developed inclusions of the GIBV, it seemed likely that the RNA existed as a contaminate which was passively incorporated into the inclusions during development.

Using the modified Lowry technique it was found that the inclusions consisted of 80.2% protein, 5% of which was associated with the virus particles. The inclusions also consisted of 0.6 to 1.9% polysaccharide, as determined by the anthrone reagent method (Morris, 1948), and about 13% lipid, as determined by gravimetric assay. The polysaccharide fraction probably occurred as a contaminate and the lipid fraction was considered to be associated with the virus membranes.

Histopathology of Infections by the GIBV

Infections by the GIBV were restricted to the fat body cells. Infected fat bodies were hypertrophied and, in terminally infected grasshoppers, appeared as irregular, flaccid, grayish masses. Initial

infections were limited usually to one or several localized parts of the fat bodies. In later stages the infections were distributed throughout the tissue. As a result, the degree of infection and hypertrophy varied in different parts of the tissue during pathogenesis.

Examinations of sectioned tissues prepared for light microscopy showed that both nuclei and cytoplasm were infected. The nuclei of cells considered to be in early stages of infection were hypertrophied and appeared less granular than nuclei of other cells. Frequently, adjacent cells contained a few inclusions which varied in size while others were filled with inclusions which were uniformly large in size. This indicated that the inclusions increased in size during infection.

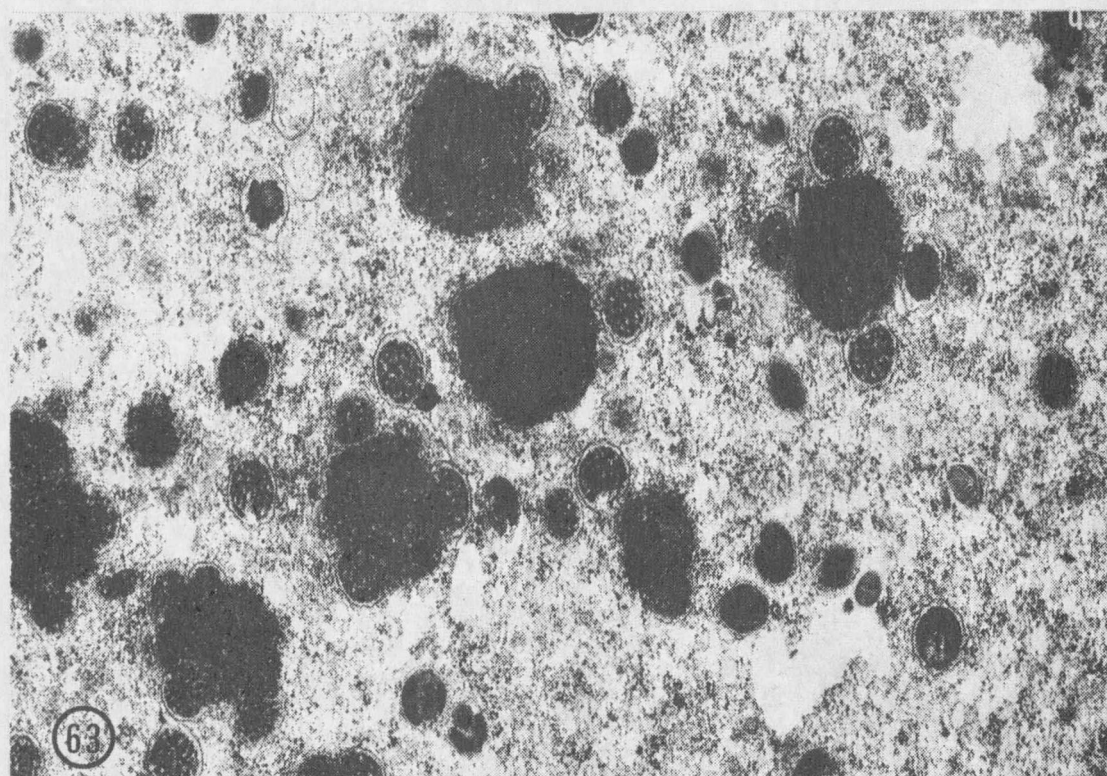
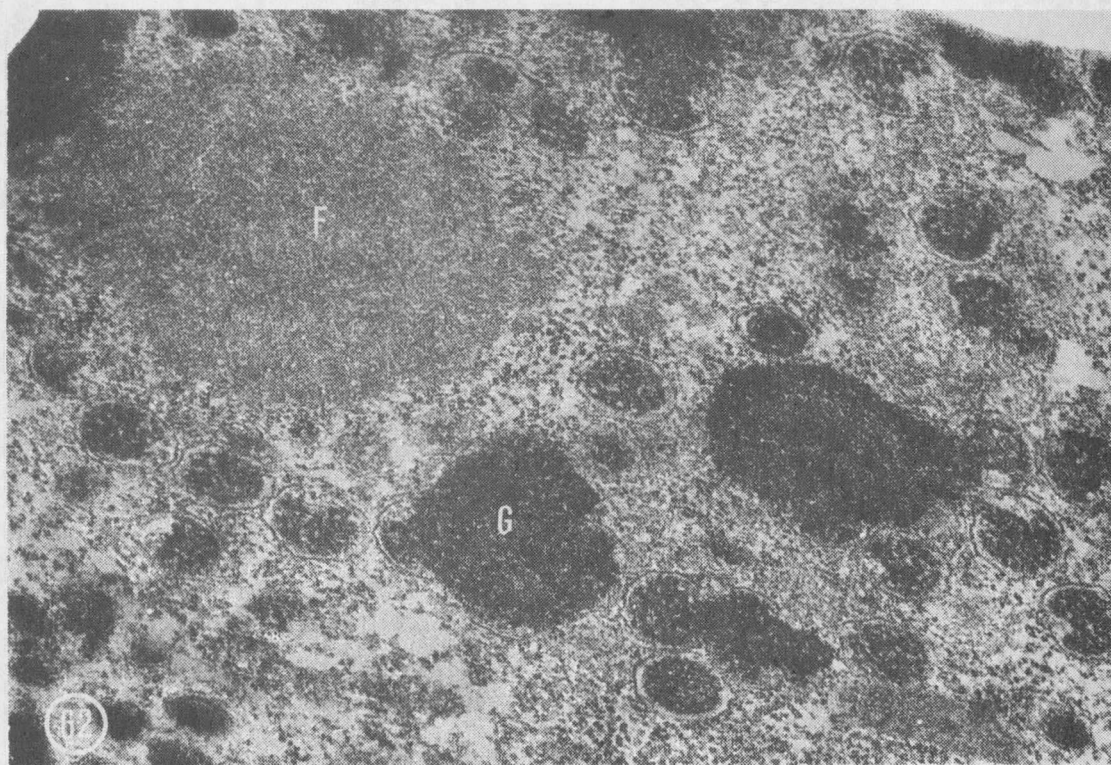
Inclusions were observed in smears of hemolymph, apparently because of lysis of heavily infected fat body cells. Because of this, the hemolymph often appeared cloudy. Inclusions were observed intracellularly in hemocytes, but this was considered due to the phagocytic activity of some hemocytes.

Replication of the GIBV

Because of the asynchrony in infections and virus development within both individual and adjacent cells, ultra-thin sections prepared for electron microscopy often contained cells which exhibited different structures involved in viral replication. The first recognizable stages, observed in sections prepared eight days after injection, were large, slightly dense masses which exhibited both granular and fibrillar characteristics (Fig. 62). In sections prepared after eight days other masses

Fig. 62. Ultra-thin section of a fat body cell of Melanoplus sanguinipes showing fibrillar-granular (F) and granular (G) precursor masses, and presumptive viral particles "budding" off the granular masses as observed during infections by the GIBV. 37,280X.

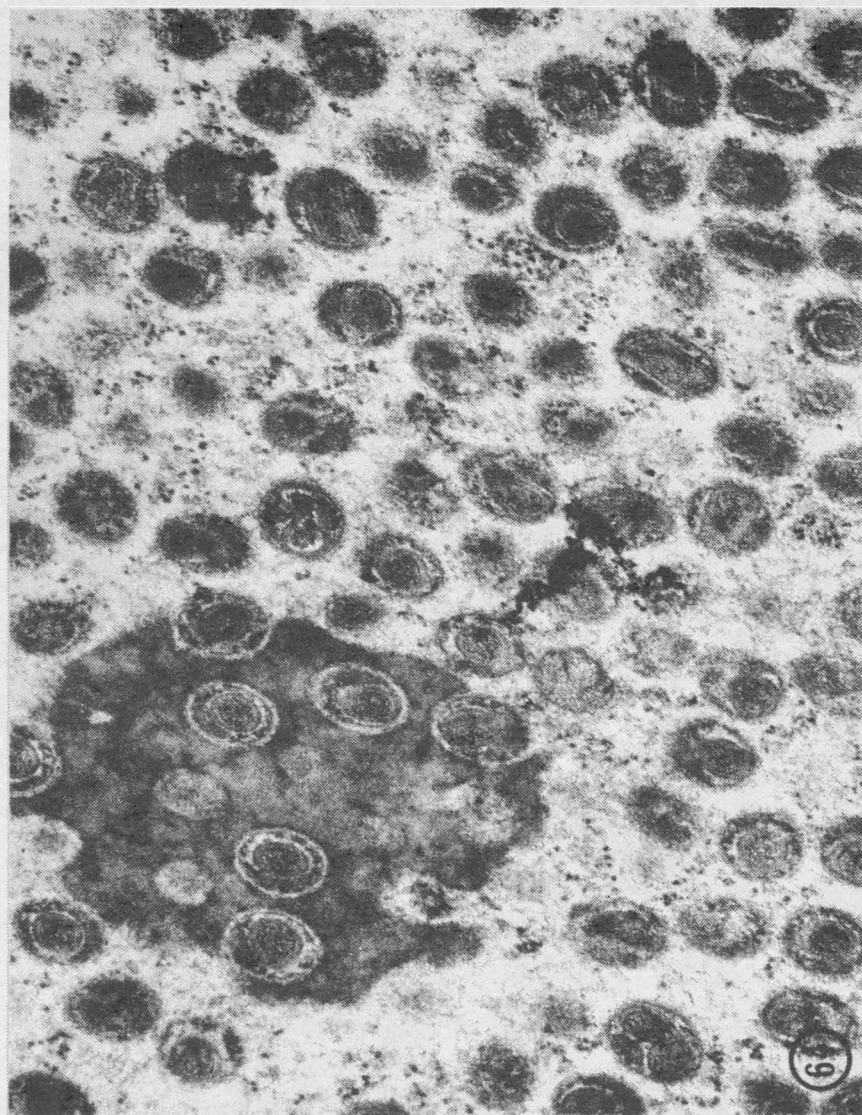
Fig. 63. Ultra-thin section of a fat body cell from Melanoplus sanguinipes showing granular precursor masses and "budding" presumptive viral particles. 32,000X.



were observed which were smaller, more dense, and characteristically more granular and less fibrillar than the larger masses (Fig. 62 and 63). Some of the smaller and more dense masses appeared entirely granular and were either partially or completely enclosed in single peripheral membranes. Apparently the smaller and more dense masses developed by condensation of the larger, less dense masses and the peripheral membranes formed from the fibrillar structures in the larger masses.

Presumptive virus particles, measuring about 320 m μ in length by about 250 m μ in width, originated as protrusions or buds from the densely granular masses (Fig. 62 and 63). These particles always appeared on the peripheral parts of the masses and were enclosed by a membrane which apparently was derived from that surrounding the granular mass. The internal structure of the particles nearest to the masses, and therefore considered the most recently developed was darkly granular but less dense than the internal structure of the precursor masses. Other smaller particles, which were considered to be of earlier origin, contained very dense granules surrounded by less densely granular areas (Fig. 63). In still other particles, these central cores or nucleoids, which appeared very dense but nongranular, were surrounded by closely associated membrane-like structures within densely granular outer coats. Although the cores of most particles at this stage appeared cylindrical, some dumbbell-shaped cores were observed (Fig. 64). Mature-appearing virus particles were observed both free in the cytoplasm and enclosed in developing inclusion bodies (Fig. 60 and 64). However, inclusion body

Fig. 64. Ultra-thin section of a fat body cell of Melanoplus sanguinipes showing viral particles and an initial inclusion of the GIBV. 32,000X.



formation did not depend on the presence of mature virus particles since immature particles were observed within the matrices of relatively small presumptive inclusion bodies (Fig. 60). Immature particles were observed in the peripheral parts of larger inclusion bodies, while the particles in the internal parts of these larger inclusions appeared mature. This indicated that particle differentiation continued during occlusion.

Gross Effects of Infections by the GIBV

Nymphs of M. sanguinipes which were experimentally infected with the GIBV exhibited general torpor, prolonged development and abnormally high mortality rates within 10 to 20 days postinoculation. The bodies of heavily infected nymphs frequently were distended with protruding cervical membranes due to the heavy accumulation of inclusions in the fat bodies. Such heavily infected nymphs rarely developed beyond the last instar in which they persisted in a semimoribund condition. Adults which developed from less heavily infected nymphs appeared weakened and rarely became sexually mature.

Because of difficulties in standardizing assay techniques, a critical analysis of the virulence of this virus was not completed. Generally, third instar nymphs of M. sanguinipes which were inoculated per os with about 10^5 inclusions failed to reach the adult stage and died between 20 and 30 days after treatment. The lethal levels of infection (number of inclusions) were about 10^5 to 10^6 inclusion bodies per mg of grasshopper weight. Injections of filtered inoculants from homogenized grasshoppers, presumably containing nonoccluded infective particles, were more active in that most

grasshoppers died in 15 to 20 days after inoculation. However, such injections resulted in fewer and smaller inclusions in the terminally infected grasshoppers. Increased mortality rates among injected nymphs were evident seven and eight days after inoculation which corresponded to the appearance of the first recognizable structures involved in viral replication. As previously reported by Henry and Jutila (1966), inclusions were discernible under light microscopy at 12 days after per os inoculations and about 10 days after injection.

Observations of the Natural Occurrence of the GIBV

During the period from 1964 to 1968, observations of the natural occurrence of the GIBV were conducted among grasshoppers collected from the Antelope Basin region in southwestern Montana. One epizootic caused by the GIBV was observed in one part of the region during the 1964 season and a second epizootic was observed during the 1966 season in a different part of the region. Although a number of different species of grasshoppers were present in the entire region, Melanoplus bruneri appeared to be the most frequently infected and was affected most by the epizootics. During the 1964 epizootic, which was detected over an area of about 15 acres during September of that year, about 57 percent of the live and nearly 100 percent of the dead M. bruneri were infected with the GIBV. Although accurate counts were not made, dead M. bruneri were abundant in the epizootic area.

The second epizootic occurred during August 1966 in an area about one mile long and 100 yards wide. Examinations of grasshoppers showed that

about 67 percent of the live and nearly 100 percent (299 of 300) of the dead M. bruneri were infected. Based on counts obtained from a series of 1-square-yard plots during a three-day period, it was determined that the M. bruneri were dying at a rate of 2.5 grasshoppers per square yard during each 24-hour period.

Although the grasshopper densities at the two epizootic locations and at other locations were not ascertained quantitatively, the species composition and estimated densities appeared altered following the epizootics. In each instance the estimated overall grasshopper densities and percentage of M. bruneri in the species composition declined during and after the epizootics while such reductions or changes were not evident at locations where epizootics had not occurred.

Effect of Host Nutrition on the Development of the GIBV

During studies with the GIBV it was noted that experimentally infected grasshoppers which were fed the artificial media contained fewer inclusion bodies than those which were fed fresh lettuce and bran. It was observed also that a change from brewer's yeast to yeast extract in the media altered the development of infections by the GIBV. A study was conducted, therefore, to quantitate the effects of these diet alterations and to determine the effect of the triple sulfa antibiotic used to inhibit Malameba locustae on virus development.

The grasshoppers used in this test were inoculated per os with 6.5×10^5 inclusions as 3rd instars. The test was terminated at 18 days postinoculation, at which time the survivors were weighed and examined.

As shown in Table VIII, the average weights of grasshoppers which were fed fresh lettuce and bran were nearly twice those of the grasshoppers in the other treatments. However, these grasshoppers also exhibited the highest incidence of infection and the highest levels of infection. They also exhibited the lowest percentage survival, which may have been caused partially by the GIBV. It was noted, however, that many of these grasshoppers died as a result of bacterial septicemia which was considered to have resulted from the excessive humidity in the rearing tubes due to the presence of the lettuce.

The grasshoppers fed brewer's yeast generally weighed more and exhibited a higher incidence of infection than those fed yeast extract. The numbers of inclusions per milligram of grasshopper weight did not differ between those fed brewer's yeast and yeast extract. It was noted, however, that the grasshoppers treated with the antibiotic contained about twice as many inclusions as those which were not treated with the antibiotic.

These results showed that the host's diet influenced its susceptibility to infections by the GIBV. Instead of the best diet, lettuce and bran, increasing the grasshoppers' resistance to disease, it apparently caused the opposite effect of increasing the susceptibility to disease. Several factors might have accounted for this. First, the grasshoppers fed lettuce and bran possibly contained more fat bodies than those fed the other diets; thus, provided more host tissue for the virus. Second, the various diets may have caused differences in the conditions within

TABLE VIII

Effect of different host diets on infection by the grasshopper inclusion body virus in Melanoplus sanguinipes.

Diet	No. grass-hoppers	Percent infected	Percent survival	Ave. grass-hoppers wt. (mg)	Level of infection* X 10 ⁵
1. Media with brewer's yeast and antibiotic	63	83.7	77.8	98.0	3.179
2. Media with brewer's yeast	44	84.8	75.0	104.9	1.841
3. Media with yeast extract and antibiotic	56	69.6	82.0	92.1	3.418
4. Media with yeast extract	50	72.2	72.0	75.1	1.864
5. Fresh lettuce and bran	45	91.7	53.3	203.4	5.609

* Level of infection is based on the number of inclusions per mg of infected grasshopper weight.

the digestive tract by changing the bacterial flora which, in turn, may have altered the release of virus particles from the inclusion bodies. Third, the various diets may have altered the physiology of the host. This latter factor may also have caused the higher levels of infection among the grasshoppers treated with the antibiotic since the antibiotic might have been slightly toxic and reduced the grasshoppers' resistance to disease.

Relationship Between the GIBV and Other Insect Viruses

The inclusions, virus particles and stages in replication appeared to be different from those of the typical inclusion body viruses described from other insects. Superficially, the GIBV resembles the cytoplasmic polyhedrosis viruses in that both develop proteinaceous inclusions. However, cytoplasmic polyhedrosis viruses contain RNA (Smith, 1963), are irregularly shaped, and have been reported from tissues lining the alimentary canal of some Lepidoptera. The GIBV resembles the nuclear polyhedrosis viruses in that both form inclusions, contain DNA, and infect the fat bodies of their hosts. However, the structure of viral particles and replication of nuclear polyhedrosis viruses, as reported by Xeros (1955, 1956) and Day, et al. (1958) and reviewed by Aizawa (1963) differ from those of the GIBV. The GIBV also differs from the DNA-containing granulosis viruses in the morphology of inclusion bodies, host tissues, and process of replication as reported by Huger and Kreig (1961) and Huger (1963).

Of the known insect viruses, the GIBV resembles most closely

viruses reported by Vago (1963) and Weiser (1965) from larvae of Melolontha melolontha (L.) (Lepidoptera) and by Weiser and Vago (1966) from the lepidopterans Operopthera brumata (Hub.) and Acrobasis zelleri Rag. The inclusions of these viruses increase in size during pathogenesis and contain randomly distributed particles which resemble those of the GIBV. These viruses infect the nucleus and cytoplasm of fat body cells. In view of these similarities it is probable that the GIBV belongs to the genus Vagoiavirus Weiser which Weiser (1965) erected for the virus from M. melolontha.

The GIBV also resembles viruses of the pox group and particularly vaccinia virus. According to Dales and Siminovitch (1961), during the replication of vaccinia presumptive particles develop from precursor granular masses after which they undergo internal differentiation to form mature particles. In addition, the electron dense central cores of presumptive particles of vaccinia appear similar to structures observed in presumptive particles of the GIBV. However, the replication of vaccinia does not involve development of inclusions and also differs in the formation of the membranes around the presumptive particles.

The Crystalline-array Virus (CAV)

The CAV was first isolated from 4th and 5th instar nymphs of Melanoplus bivittatus which exhibited an unusually high rate of mortality while being reared in the laboratory. Several days later, high rates of mortality also were noted among M. bivittatus in several other nearby cages.

These grasshoppers were being reared at 85° C. under continuous illumination and were being fed fresh lettuce, wheat bran, and the artificial diet containing the triple sulfa antibiotic for prevention of Malameba locustae. Initially, the etiological agent was characterized as a probable virus because of infectivity of supernatants of homogenates following low speed centrifugation (3700 to 4500 rpm) and filtrates which passed through millipore filters with average pore sizes of 0.45 microns.

Host and Geographical Range of the CAV

Natural infections by the CAV among grasshoppers in the field have not been observed. However, a number of Melanoplus sanguinipes, Opeia obscura and Amphitornus coloradus which were collected from CAV-treated experimental field plots exhibited symptoms associated with infections by the CAV. In the laboratory the CAV has been transmitted to Melanoplus bivittatus, M. sanguinipes, M. differentialis, M. femurrubrum, M. dawsoni, Amphitornus coloradus, Aulocara elliotti, Schistocerca vaga vaga, and S. americana. Differences have been noted in the susceptibility of some species to the CAV, specifically M. bivittatus and S. americana appeared more susceptible to infections than M. sanguinipes and M. differentialis.

Morphological and Chemical Characterization of the CAV

The disease was diagnosed microscopically by the presence of crystals in whole-body homogenates, fresh squash preparations of muscles, pericardial tissues, and tracheal matrix (Fig. 65), and sections of pericardial tissues and muscles (Fig. 66). The crystals varied in size and shape, but

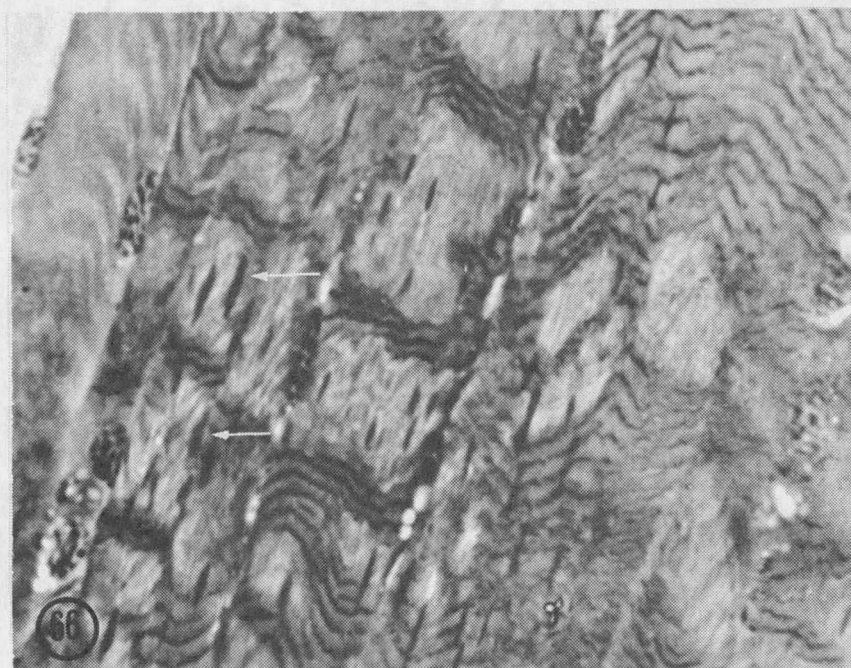
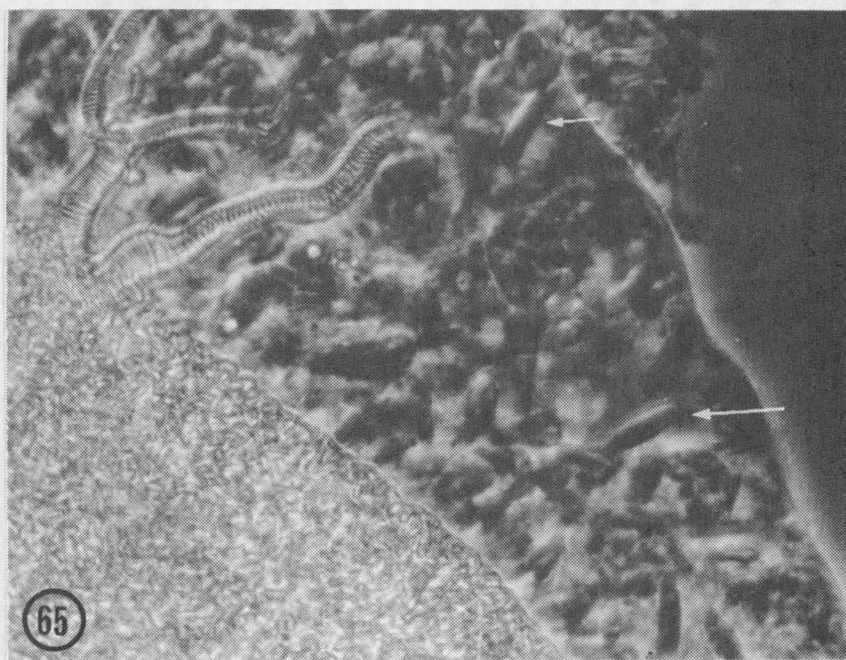
generally appeared as rods 2 to 10 microns in length with rounded ends. The crystals from grasshoppers in early stages of infection, 2 to 5 days, generally were smaller than those from grasshoppers in later stages of infection, 8 or more days.

Electron micrographs of muscles and pericardial tissues revealed the presence of cytoplasmic crystalline structures which were composed of small spherical particles (Fig. 67), each of which measured about 13 μ in diameter. In cross section, the crystalline structures varied in size and complexity and macrocrystals found in terminally infected grasshoppers frequently exhibited crystalline arrays oriented in several different planes (Fig. 68).

Three major light scattering zones appeared in 10%, 20%, 30%, 40% sucrose gradients following centrifugation for 3 hours at 2° C. and 35,000 rpm in a Beckman model L ultracentrifuge with an SW-39 rotor. The top zone appeared at 0.5 to 0.7 cm below the meniscus, the middle zone at 1.7 to 1.9 cm below and the bottom zone at 2.7 to 2.9 cm below. The middle and bottom zones failed to appear with tissue extracts from noninfected grasshoppers. The top zone contained abundant cellular debris and, in infected grasshopper extracts, numerous crystalline structures judged to be of viral origin. The middle zone predominately contained small particles, 13 to 15 μ in diameter, with increasing numbers of small crystals or aggregates when more concentrated homogenates of virus-rich tissues were used. The bottom zone consisted of aggregates of various sizes which dissociated when resuspended in a larger volume of Tris^{9/}

Fig. 65. Fresh preparation of the tracheal matrix of a Schistocerca americana containing crystals of the CAV (arrows). 1500X.

Fig. 66. Ultra-thin section under light microscopy showing crystals (arrows) of the CAV in the muscles of Schistocerca americana. 1200X.

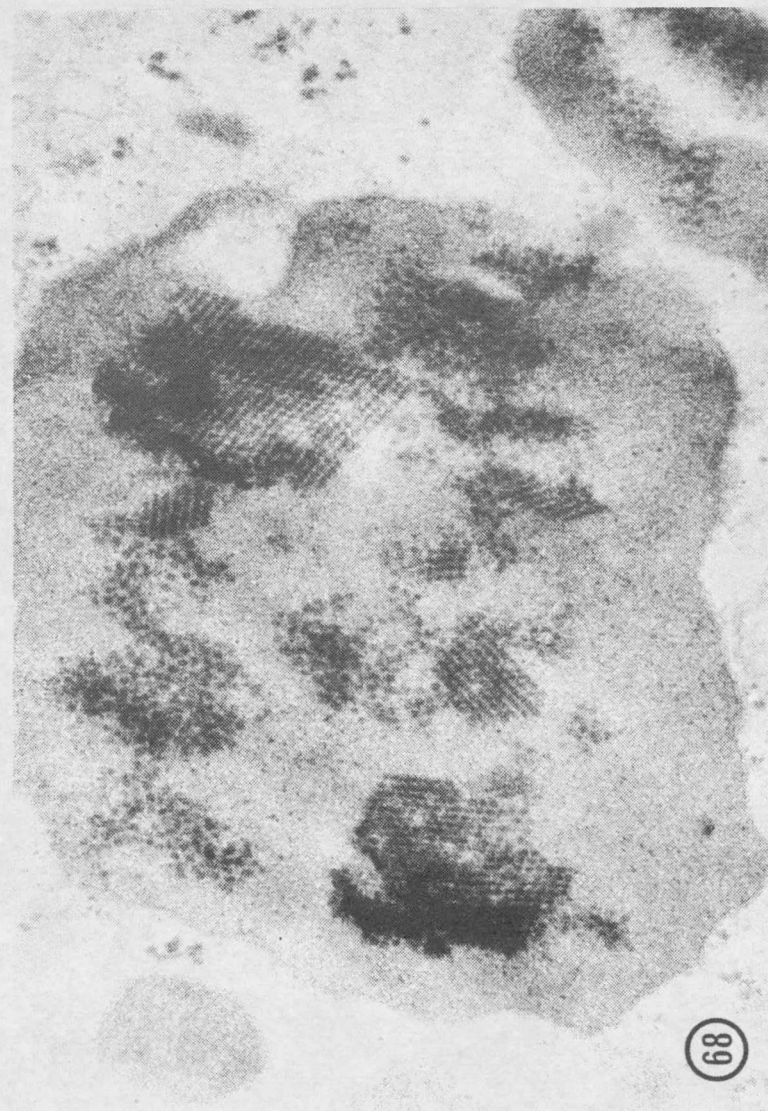
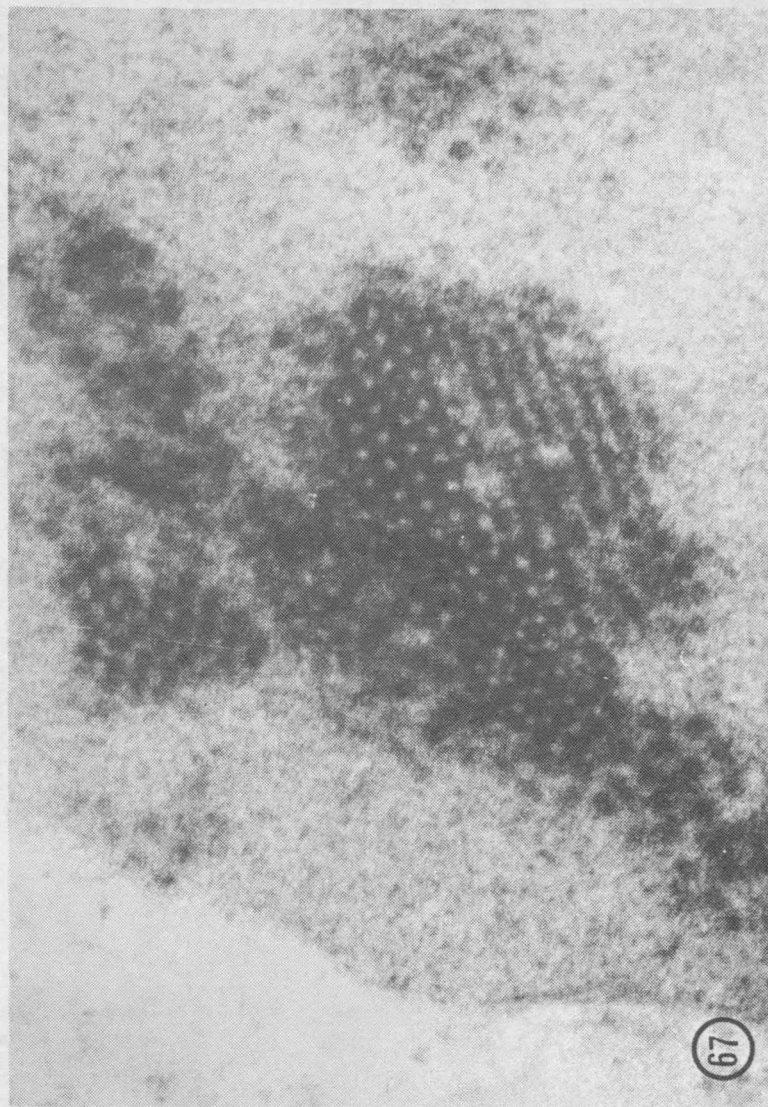


-125-

Fig. 67. Ultra-thin section of a pericardial cell of Schistocerca americana showing the crystalline array arrangement of viral particles in a developing crystal. 205,000X.

Fig. 68. Ultra-thin section of a pericardial cell showing developing crystals of the GAV in an electron dense replicating matrix. 66,000X.

duplex
25% cotton



buffer (pH 6.4-6.7) and upon recentrifugation in a sucrose gradient. Examinations of the Schlieren patterns obtained during ultracentrifugation in Beckman model E analytical centrifuge showed that the middle zone formed a major virus peak which was preceded by a more rapidly moving and increasing peak. This indicated that the free virus particles were aggregating during centrifugation and moving into the faster moving peak which formed the bottom zone in the sucrose gradients.

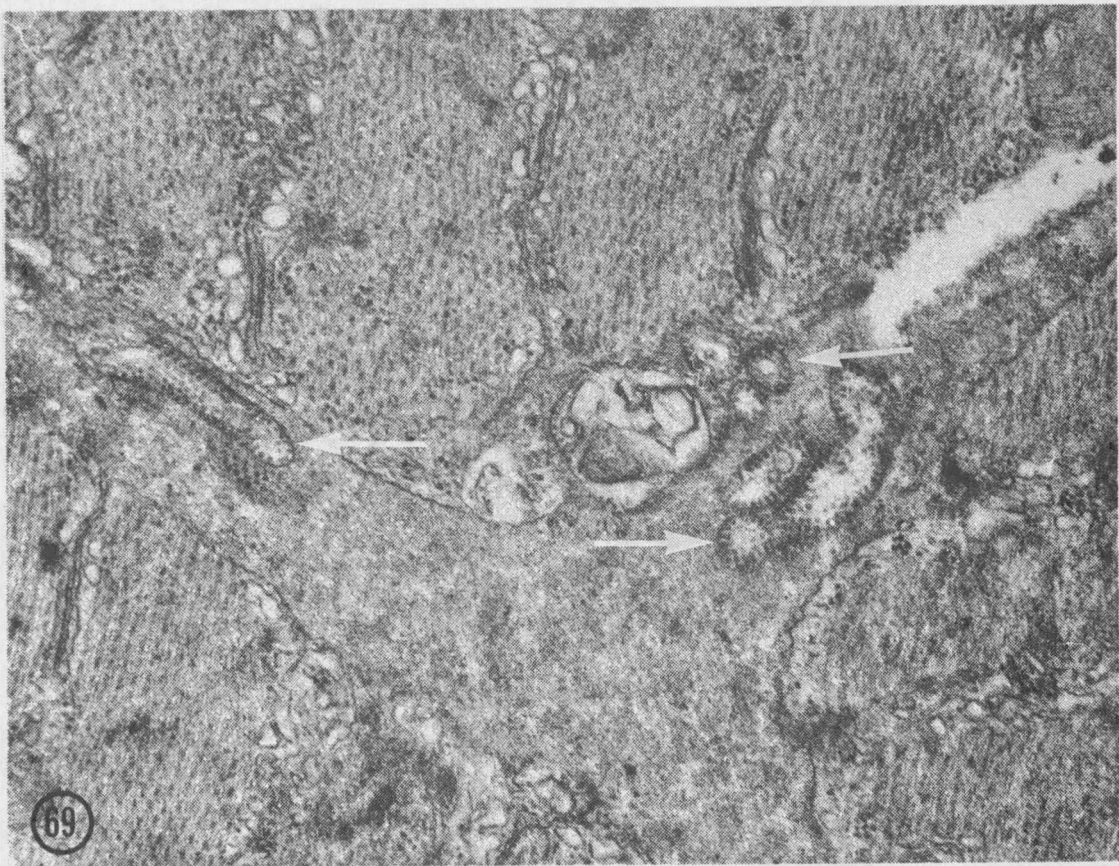
Chemical analysis of the purified virus from the middle zone showed it consisted of 18 to 22% RNA and 78 to 82% protein. Assays for carbohydrates, DNA and lipid consistently were negative.

The virus was unstable in phosphate buffers at pH 5.6 or below and above 8.6 or in suspensions at room temperature for 4 days. The virus was infective after 7 months storage in distilled water at about -4° C.

Replication of the CAV

Electron micrographs of muscle tissues from infected grasshoppers revealed the presence of vesicular membrane-like structures with peripherally oriented small particles presumed to be viruses (Fig. 69). Loosely arranged virus particles also were observed within the lumen of the vesicles and in others they were arranged in small crystalline arrays. Poorly defined coalescing membranes were observed around larger crystals and virus particles were observed in the areas between the membrane and crystal. The matrix within the membranes and around the crystals and particles appeared more electron dense than the cell cytoplasm outside the vesicles. These observations indicated that virus particles

Fig. 69. Ultra-thin cross section of the muscles of Schistocerca americana containing several vesicular membrane-like structures (arrows) encircling viral particles. 59,000X.



replicated on the interior surface of the membranes and then aggregated in the lumen to form the crystalline arrays. Generally the crystals developed such that their long axis was parallel with the muscle fibers.

Virus replication in the pericardial cells appeared similar to that observed in the muscles except the electron dense replicating areas were larger and often contained several crystalline arrays which were oriented in different planes (Fig. 69). The vesicular membrane surrounding the electron dense area was less well defined in pericardial tissues.

Gross Effects of Infections by the CAV in Grasshoppers

Schistocerca americana generally exhibited initial symptoms of vigor and coordination within 4 days after experimental inoculation. Most mortalities occurred between 6 to 10 days postinoculation and moribund grasshoppers were unable to remain upright. The thoracic muscles of moribund grasshoppers lacked the shiny and elastic characteristics of muscles of uninfected grasshoppers. Instead they appeared granular and were easily separated with slight upward leverage. Examination of prepared sections of muscles showed that most crystals were located at the ends of the muscle fibers. Spheroid blood cells and pericardial cells were frequently observed between the muscle fibers from moribund grasshoppers.

During the initial studies of the CAV, a number of assays were conducted with results which usually were consistent with the assay results shown in Table IX. These data were derived from the number of mortalities among 3rd instar nymphs of Melanoplus bivittatus and M.

TABLE IX

Cumulative percent mortality among third instar nymphs of Melanoplus bivittatus and M. sanguinipes after per os inoculations with CAV.

Day after inoculation	Dilution of infected homogenate ^{a/}			Controls
	undiluted	10 ⁻¹	10 ⁻²	
	(33) ^{b/}	(30)	(22)	(55)
4	25.0	36.7	13.6	18.2
5	66.6	56.7	36.4	32.8
6	70.8	73.3	40.9	34.5
7	79.2	80.0	40.9	34.5
8	91.7	83.3	45.9	34.5

a/ Each treated grasshopper was fed 5 μ l of infected tissue homogenate at day 0.

b/ The number in parenthesis designates the number of grasshoppers treated in each group.

sanguinipes which were individually inoculated per os with 5 μ l of an homogenate obtained by suspending one nymph of M. bivittatus in 10 ml of distilled water. These data indicate that infectivity was demonstrable at 100-fold dilutions. However, intrahemocoelic injections of 5 μ l of purified virus suspensions containing 17 to 63 μ gm of virus protein consistently produced mortalities at 10^{-5} dilutions. Intrahemocoelic injections generally caused comparable mortalities at 10-fold greater dilutions as did per os inoculations. In addition, 3rd instar nymphs appeared more susceptible to the effects of infection than did older nymphs and adults.

Similarities Between the CAV and Other Viruses

The small size and RNA content of the CAV indicated a relationship with viruses of the picornavirus group. However, this relationship has not been confirmed since it has not been determined whether the RNA of CAV is single-stranded or double-stranded. Also, because of the small size and orientation into crystalline arrays, the CAV is unlike other RNA-containing viruses of insects. The RNA-containing virus isolated by Meynadier (1966) from the cricket Gryllus bimaculatus Geer apparently is more than twice the size of CAV and does not aggregate into crystalline arrays. In repeated tests, the CAV was not infective when inoculated into eastern house crickets, Acheta domesticus.

DISCUSSION AND CONCLUSIONS

These studies have shown that the protozoan and viral pathogens of grasshoppers vary from those which are highly virulent and cause increased rates of mortality, as observed among individually reared grasshoppers, to those which cause no apparent effect on the host and, therefore, might be considered as avirulent. The Eugregarinida appeared to be the least virulent of the reported pathogens, followed in sequence of increasing virulence by Malameba locustae, Nosema locustae, the grasshopper inclusion body virus (GIBV), the undescribed species of Neogregarinida, the Nosema sp., Nosema acridophagus, and the crystalline-array virus (CAV). Because of the lack of information, the small spore organisms have not been included in this scheme; however, preliminary observations have indicated that their virulence is comparable to that of N. locustae or M. locustae.

Based on the laboratory studies of these organisms, one of the factors responsible for the relative virulence exhibited by a pathogen is which host tissues are infected. For example, the two least virulent pathogens, Eugregarinida and Malameba locustae, infect the epithelium of the digestive tract and, in the case of M. locustae, the epithelium of the Malpighian tubules. By comparison, the pathogens which infected and destroyed the fat bodies of grasshoppers, such as Nosema locustae and the GIBV, appeared more virulent, while those which infected a number of tissues, such as the Nosema sp., N. acridophagus and the CAV, appeared to be the most virulent. The low virulence exhibited by the Eugregarinida and M. locustae probably is due to the normal replacement of epithelial cells of the midgut and gastric caeca by cells from the regenerative nidi

and by the supplemental excretory function by the hindgut, fat body, and pericardium. The higher virulence expressed by the pathogens of the fat body in relation to those which infect the digestive tract epithelium is contrary to the generalizations made by Weiser (1961) and Kramer (1963) that pathogens of the digestive tract are more virulent than those of the fat body of insects. They based their ideas on the premise that the fat body is relatively inert and functions primarily in storage. However, according to Munson (1953), in addition to a storage function, the fat body carries out metabolic processes which are vital to the life of the insect. The mortalities caused by the GIBV and N. locustae indicate that the fat body is definitely vital to the survival of grasshoppers.

Another factor which appeared responsible for the relative virulence exhibited by a pathogen is the rate at which the pathogen increases. This was demonstrated by the fact that the two most virulent pathogens, N. acridophagus and the CAV, required the shortest time between infection to the appearance of new infective stages. Crystalline aggregates of CAV were observed within four days after inoculation while spores of N. acridophagus were usually observed in 4 to 6 days postinoculation, and, as previously mentioned, spores also were observed in tissues which were sectioned at 48 hours after inoculation.

The indication that the more virulent pathogens required a shorter period of time between inoculation and the appearance of new infective forms was evident in the study of the susceptibility of Melanoplus bivittatus to Nosema locustae, N. acridophagus, and the Nosema sp. As

shown in Fig. 53, N. acridophagus increased to lethal concentrations at about 10 days postinoculation, which corresponded to about twice the length of its developmental period (normally 4 to 6 days). Similarly, the Nosema sp. caused increased rates of mortality at about 18 days postinoculation which also corresponded to about twice the length of its developmental period. However, the inverse relationship between the relative virulence expressed by a pathogen and the levels of infection observed among the individually-reared grasshoppers (Tables III, VI, VII) indicated that despite the shorter developmental period and increased virulence, the more virulent microsporidians ultimately produced fewer spores and, therefore, probably would have, under natural conditions, a lower frequency of transmission. In effect, this indicates a probable adaptation in the host-pathogen relationship which permits the continued existence of both.

A third factor which functions in the pathogenesis of each of these organisms is the response of the host to the infection. Although insects lack specific immune responses (Stephens, 1963), phagocytic activity by grasshopper hemocytes was observed. Also, the tumors observed during infections by N. acridophagus appeared to be cellular responses against the dissemination of the organism. The encapsulation of spores in the melanotic cores of the tumors indicated a reaction by the host which not only inhibited the proliferation of the organism throughout the body of the host, but also decreased the number of spores available for subsequent transmission. The encapsulation of Malpighian tubules which were compacted with cysts of Malameba locustae appeared to be a similar process which

prevented the liberation of cysts into the hemocoel. There were indications from the differences in sizes of cysts from a grasshopper with encapsulated tubules that the encapsulation process weakened the cyst walls and possibly caused decreased viability.

A fourth factor which influenced the relative virulence of a pathogen was the effect of secondary factors on either the pathogen itself or on the host. As observed in the study of the susceptibility of Melanoplus bivittatus to the microsporidians, the condition of group-rearing had an effect of increasing the virulence associated with each pathogen. This was evident from the high rates of mortality during the first 10 days postinoculation among the grasshoppers inoculated with each of the microsporidians (Fig. 54). However, these mortalities were not due to the action of the microsporidians, since none of the pathogens had increased to lethal levels during this first 10-day period, but were due instead to the combined effect of cannibalism and infection. The high mortality rate among the untreated grasshoppers (38.8%) indicated that cannibalism occurred at a high frequency among the untreated grasshoppers; however, the even higher rates among the treated grasshoppers indicated that cannibalism was more frequent among these. In effect, the diseased condition probably could be considered as a predisposing factor for mortalities due to cannibalism. Also, it was noted that rate of mortality among group-reared grasshoppers infected with the least virulent organism, N. locustae, was similar to that of the most virulent species, N. acridophagus. The differences in virulence of the pathogens becomes less

evident in the presence of other factors.

As observed in studies with the GIBV, the host's nutrition also influences its susceptibility to disease. However, in this case the grasshoppers fed the best diet, as indicated by increased weights, appeared more susceptible to the virus. This points out that because of the lack of effective immune responses, the increase of the pathogen is dependent on the availability of suitable host tissues. It seems probable in this study that the grasshoppers fed the best diet also possessed more fat bodies than those fed the other diets.

In addition to the direct effects of infections, as exhibited by increased rates of mortality, reduced weights, etc., the pathogens of grasshoppers cause secondary effects which frequently are expressed by reduced reproduction. For example, infections by N. locustae reduced the number of egg pods produced by females of Melanoplus differentialis and, although quantitative studies were not conducted, adult females which were infected with the GIBV rarely contained fully developed ovaries which indicated that reproduction either was completely or at least partially inhibited. In addition, Prinsloo (1962) reported that the antibiotic factor present in locusts which were infected with Malameba locustae destroyed the normal bacterial flora of the ovaries and this resulted in a changed diapause pattern in the development of the eggs. As a result, the viability of the eggs and offspring was decreased. This may be related to the findings by Jackson et al. (1968) of alterations in the lipid composition of eggs from infected females. Possibly the loss of the normal

Klebsiella flora in the females resulted in the different lipid content of their eggs and, in turn, caused the reduced viability.

Under natural conditions pathogenic organisms generally are uncommon among grasshoppers. This has been evident in the results of the study of the natural occurrence of Nosema locustae in grasshoppers in Camas County, Idaho. Even though this area was selected for study because of the unusually high incidence of N. locustae, only about 5% of the more than 30,000 grasshoppers examined during the 6 years of the study were found to be infected. In addition, most of these infections occurred in grasshoppers collected from three chronically infected locations within the study area where the incidence and levels of infection generally were consistent from one season to the next, despite fluctuations in grasshopper densities and species composition. Usually, at these locations, about 5% of the grasshoppers were infected by the latter part of July and from 40 to 80%, and as much as 95%, were infected by the latter part of September or early October. Throughout the major portion of the study area the incidence of infection averaged less than 3% but occasionally increased to 10 to 20% at specific sites by the end of the season.

N. locustae was much less common, as generally were other pathogens, among grasshoppers which were collected at other locations in western United States where there was no knowledge of prior infections. However, these collections have indicated that the natural incidence of infection varies inversely with relative virulence of the specific pathogen. For example, the eugregarines have been the most common pathogens observed in

these collections. Quite frequently between 30 to 70% infestations by eugregarines were observed during the latter part of the summer season. However, such infestations generally were confined to only a few of the species present in a localized area. The eugregarines were the most common organisms observed in grasshoppers collected from the San Carlos Indian Reservation in Arizona, followed in order by Malameba locustae, Nosema locustae, and the GIBV. In Camas County, Idaho, Nosema locustae was the most common organism followed by the GIBV and the neogregarine. Nosema sp. has been observed only in grasshoppers collected from the one location near Bozeman, Montana. Although the incidence of Nosema acridophagus among grasshoppers in southeastern United States has not been determined, this pathogen has never been observed in grasshoppers collected in western United States. The CAV has never been observed under natural conditions and, therefore, there is no evidence that it exists as a pathogenic agent in nature.

This relationship between relative virulence and natural occurrence of a pathogen, together with such factors as increased infections during the latter part of the season, means of pathogen transmission, rate of pathogen increase, etc., suggests a natural equilibrium between the pathogens and grasshoppers. In effect, it is apparent that grasshopper and pathogens have become adapted to a relationship which insures the survival of each. Because of this equilibrium, the pathogens rarely increase to levels where they destroy the host population; a situation which would be as detrimental to the pathogen as the host. It is

conceivable that a high incidence of infection may occasionally be beneficial to both the pathogen and host. For example, a high incidence of infection, or even 100% mortality, during the latter part of the season would have no effect on the survival of the host population because most eggs would already have been produced. A drastic reduction in the density after that period, however, might provide more food for the next generation of hosts. At the same time, increased pathogen numbers during the later part of the season increases its own survival potential.

According to Andrewartha and Birch (1954), Thompson (1956), Solomon (1957), Nicholson (1958), and others, the density of animals, and particularly insects, is determined by the interaction between organisms and various factors in the environment. Although these authors disagree on the mechanism of action, all are agreed that some sort of balance or equilibrium exists between the environmental factors and insects so that as the effect of one factor in the environment changes the effects of all other factors are subsequently altered. They all consider disease as one of the factors which functions in the regulation of insect densities.

According to Steinhaus (1958) diseases of insects appear to be density dependent factors, which means in effect that as the density of the host increases the percentage of infection also increases. He postulated that epizootics caused by pathogenic microorganisms would occur most frequently during periods of high host densities. Apparently this happens not only because of the increased probability of disease transmission during periods of high host densities, but also because the

high densities act as stress factors which increases the susceptibility of the insects to disease.

The interjection of stress factors, or predisposing factors, into the host-pathogen relationship serves to complicate the situation because it becomes difficult to separate cause and effect in the disease process. Assuming that the densities of the host are determined by the interaction of environmental factors, it is conceivable that a high incidence of disease may be induced by changes in the effects of other factors or possibly a high incidence of disease may act as predisposing factors for increased effects by other factors.

It is not known whether or not grasshopper pathogens are density dependent factors or even the extent to which they are acted on by, or act as, predisposing factors. However, high incidence of disease has been observed under natural conditions in which case the pathogens have appeared capable of or responsible for reductions in host densities. For example, numerous dead grasshoppers, heavily infected with Nosema locustae, were collected during the study in Camas County, Idaho. Most of these occurred late in the season; however, some heavily infected dead grasshoppers were collected during July and August indicating that N. locustae is potentially capable of reducing host densities.

The studies of the incidence of the GIBV among the Melanoplus bruneri in the Antelope Basin region in Montana showed that this virus is capable of killing grasshoppers in naturally occurring situations. The reduced densities of M. bruneri following the epizootics indicated that the GIBV

functioned as a regulating factor. The report by Venter (1966) that infections by Malameba locustae prevented an expected outbreak of the brown locust also showed that this pathogen functioned as a regulating factor. However, in all cases, including the studies of infections by Nosema locustae in the grasshoppers in Camas County, Idaho, the epizootics were limited to the grasshoppers in particular locations where, apparently, the natural equilibrium between the host and pathogens was altered. This poses the question of what caused the changes in this equilibrium. Although data are not available to answer this question, several possible explanations might be postulated. First, there may have been more disease causing organisms available at these locations. Second, some factors in the environment were altered to the benefit of the pathogen. Third, some physiological factor or factors of the host were altered such that the host was more susceptible or predisposed to the pathogen, or fourth, a combination of the first three factors.

As with many other studies of the diseases of insects, these studies have been directed at the ultimate use of pathogens for controlling grasshopper densities. In view of the foregoing, it appears that pathogens are capable of controlling grasshopper densities either by increasing the rates of mortality, reducing the reproductive levels, or by increasing their susceptibility to other controlling factors. Potential control of grasshoppers is based on the idea that disease is one factor which regulates grasshopper densities and that the effect of disease can be increased by altering the natural equilibrium. The primary factor to

be altered in this equilibrium is the availability of disease. For example, it is probable that by artificially disseminating the pathogens during the early part of the season, particularly while the grasshoppers are in the nymphal stages, there will be an increased incidence of disease during the early adult stages which will then provide a basis for an even higher incidence during the ovipositional period. As a result there should be fewer grasshoppers to deposit eggs and probably a decreased viability in the eggs and offspring which would subsequently result in lower host densities.

SUMMARY

These studies showed that the protozoan and viral pathogens of grasshoppers varied in their effect on the host from those which caused no apparent effect to those which caused increased rates of mortality within 10 days after experimental infection. The Eugregarinida appeared to be the least virulent, followed in sequence of increasing virulence by Malameba locustae, Nosema locustae, the grasshopper inclusion body virus (GIBV), an undescribed species of Neogregarinida, an undescribed species of Nosema, Nosema acridophagus, and the crystalline-array virus (CAV). There was not sufficient information available to place the small-spore-type organisms, which are probably species of Microsporidia, in this scheme. Preliminary observations indicated, however, that their virulence was comparable to that of N. locustae and M. locustae.

Infections by the Eugregarinida resulted in no apparent effect on the hosts and were diagnosed only by the detection of the eugregarine. They were specific for the epithelial tissues in the gastric caeca and midgut and their low virulence apparently resulted from the normal replacement of spent epithelial cells by cells from the regenerative nidi. Conceivably, infections by eugregarines caused increased rates of cell replacement which might secondarily have interfered with other body functions; however, this was not expressed through any observable gross symptoms.

Infections by Malameba locustae appeared somewhat more virulent than those by eugregarines in that the infected grasshoppers were less active than normal and occasionally exhibited increased rates of mortality.

M. locustae infected the epithelial tissues of the midgut, gastric caeca and Malpighian tubules, but the infections did not appear detrimental to the host because the cells from the regenerative nidi replaced epithelial cells and other tissues supplemented the excretory function of the Malpighian tubules. However, a toxic factor occurred in infected grasshoppers which exhibited bacteriostatic activity and toxicity to grasshopper embryos. Possibly this toxin was responsible for observed histolysis of muscles and fat bodies and ultimately for the reduced activity, increased mortality and reduction in fat body tissues.

Nosema locustae occurred primarily as a pathogen of the fat bodies of grasshoppers. The pathogenesis of infections by N. locustae apparently involved the competition between the host and pathogen for the fat body tissues. The infections were diagnosed by the behavior and appearance of heavily infected grasshoppers and by the gross changes in the appearance of the fat bodies. Grasshoppers which were inoculated as 3rd instar nymphs with 10^5 spores usually exhibited increased rates of mortality at about 30 days postinoculation.

The GIBV was specific for fat body tissues and appeared slightly more virulent than N. locustae in that fat bodies of moribund and dead grasshoppers infected with the GIBV were not as heavily infected and not as completely destroyed as were those in moribund or dead grasshoppers infected with N. locustae. Infections by the GIBV occurred in both the nuclei and cytoplasm of fat body cells while N. locustae infected only the cytoplasm. Generally, 3rd instar nymphs of M. sanguinipes which were inoculated with

10^5 inclusions exhibited increased rates of mortality within 20 days postinoculation.

The factors responsible for the virulence expressed by the neogregarine and Nosema sp. were not determined. However, the pathogenesis of infections by the neogregarine probably involved concurrent infections in the digestive tract and fat bodies. The virulence exhibited by Nosema sp. probably resulted from this species' ability to infect a number of tissues, and particularly from infections in the neural tissues.

N. acridophagus also infected many host tissues, but its relatively high virulence resulted mainly from the induction of tumors. The tumors which developed in the midgut and gastric caeca disrupted the tissues and caused lesions in the basal membrane. As a result, many of the infected grasshoppers died from bacterial septicemia.

The behavior of moribund grasshoppers infected with the CAV indicated that loss of motor function was involved in pathogenesis. This probably resulted from infections in muscles, particularly the thoracic muscles. The infections in the tracheal matrices might also contribute to the high virulence associated with CAV; however, infections in the tracheal matrices were not as consistent as were those in the muscles.

FOOTNOTES

- 1/ Jutila, J. W., Department of Botany and Microbiology, Montana State University.
- 2/ Cerophyl Laboratories, Inc., Kansas City, Missouri.
- 3/ Clay-Adams, Inc., New York, New York.
- 4/ Aloe Scientific, St. Louis, Missouri.
- 5/ Carl Zeiss, Inc., New York, New York.
- 6/ Acufine, Inc., Chicago, Illinois.
- 7/ R. P. Cargille Laboratories, Inc., Cedar Grove, New Jersey.
- 8/ The Chlorox Company, Oakland, California.
- 9/ Trishydroxymethyl amino methane (Tris). Nutritional Biochemicals, Cleveland, Ohio.

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