DEFENSE MECHANISMS OF THE CORN EARWORM, *HELIOTHIS ZEA* (BODDIE), AGAINST INVADING MICRO-ORGANISMS

By

PETER YEE-KONG CHEUNG

Bachelor of Science

Oklahoma Baptist University

Shawnee, Oklahoma

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Thesis Approved:

Thesis Adviser

Dean of the Graduate College

977034

PREFACE

This study is concerned with the possible defense mechanisms of the corn earworm, *Heliothis zea*, against bacteria injected directly into the haemolymph. Both cellular and humoral responses were under investigation. The primary objective was to determine their possible existence and relationship to each other.

I wish to express appreciation to my major adviser, Dr. E. A. Grula, for his guidance and patience during the preparation of this thesis. Appreciation is also expressed to Dr. R. L. Burton, US Department of Agriculture, for his supply of experimental insects and his advice on maintenance of these insects in the laboratory.

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CHAPTER I

INTRODUCTION

Attention to the defense mechanisms of insects and other invertebrates, far from being a modern fad, dates from the classical works of Metchnikoff, Cuénot, Metalnikov, Paillot, and Cameron in the early 1900s. Much of their work has been discussed in earlier reviews by Alekseyev (2), Briggs (7), Stephens (34), and Whitcomb et al. (42). Today, and increasingly over the past several decades, immunologists have become interested in invertebrates other than insects. It is widely believed that production of specific antibody-like gamma globulin is a function of the lymphoid tissues of higher vertebrates, but it does not occur in lower vertebrates nor invertebrates. Failure of researchers to detect the presence of such molecules, as well as failure to demonstrate accelerated clearance rates after secondary challenge of antigens reinforces such a belief. Nevertheless, while agreeing that conventional antibodies are lacking in invertebrates, immunologists have not been discouraged from seeking other forms of recognition factors by which an organism could distinguish "self" and "non-self" elements.

In 1901, Metalnikov began his study of the natural immunity of the greater wax moth against the tubercle bacillus

and showed that these bacilli were first phagocytized by "leukocytes" (same as hemocytes or haemocytes for a more modern terminology) in the haemocoel (42). Later, Metalnikov and his co-worker at the Pasteur Institute extensively studied immunity in Insecta. In 1933, Metalnikov concluded that the defense reactions of insects exhibit five principal manifestations: (1) phagocytosis, (2) giant cell formation, (3) capsule formation, (4) bacteriolytic secretions, and (5) antibody formation. The first three manifestations are often referred to as "haemocytic reactions" or "cellular immunity."

Phagocytosis or the engulfment of foreign bodies by cells is a widely distributed property in the animal kingdom. It has been reported in various phyla such as Protoza (40), Porifera (10), Echinodermata (16, 17), and Mollusca (14, 36); data on phagocytosis by insect haemocytes are extensive (3, 19, 30, 31, 40, 41, 44).

The studies of Metalnikov on phagocytosis suggested that (1) there are microbes that provoke an extremely rapid reaction of short duration and (2) phagocytosis occurs during both lethal and non-lethal infections. When a pathogen possesses low virulence and phagocytosis within the insect occurs, a fatal infection is unlikely. When a pathogen of high virulence gains access to the haemocoel, multiplication of the pathogen may exceed phagocytic capacity, or the phagocytes can be destroyed by some toxin (or toxins) of the invading organisms. Thus phagocytosis is not always a reliable method to rid the haemocoel of invading micro-organisms.

In contrast to Metalnikov, Andre Paillot concluded that insects possess both cellular and humoral defense mechanisms. He stated that many microbes, under certain conditions, were capable of provoking responses which he felt were probably associated with the formation of antibodies. Today, it is no longer believed that true antibodies are produced by invertebrates, but the relative importance of a humoral type of response is still a matter of conjecture (2, 7, 33, 42). According to Paillot, haemocytes, in addition to their phagocytic role, may act in other ways such as the extra-cellular release of bactericidal substances, resulting in non-specific or even specific resistance. Recently, the importance of humoral factors in the defense of insects against invading micro-organisms has received wide recognition. Although the existence of natural antimicrobial substances and induced resistance in insects have not been definitely demonstrated, researchers have reported the discovery of various natural and/or acquired forms of resistance (22, 42). The difficulties involved in demonstrating such mechanisms have been emphasized by Boyden (6).

In summary, the immediate question to be answered is: Given that a cellular response (phagocytosis) is provoked by direct injection of bacteria into the haemocoel of the corn earworm, are additional phenomena involving haemolymph proteins involved as a humoral response of the insect?

CHAPTER II

MATERIALS AND METHODS

The Corn Earworm Colony

Corn earworm larvae used in this study were reared on CSM diet at room temperature, as described by Burton (8,9). Individual, newly-hatched larvae were placed in diet cups and maintained under conditions of high humidity. When larvae reached the third instar (about 8 to 9 days), they were examined and weighed. Healthy-looking larvae, weighing about 150 mg, were selected for the various experiments.

Blood Sample Collections

Two methods of blood collection were employed. First, when a larva was not needed for further tests, it was punctured on the back close to the head. A drop of fresh haemolymph would form, which was then either collected into a capillary tube pre-coated with phenylthiourea (to prevent melanization), or dropped onto a clean slide. Second, when more tests were to be run employing the same larva, it was punctured at the base of its proleg. A drop of haemolymph would then form on the skin of the larva, which could readily be collected into a phenylthiourea-coated capillary tube by capillary action. Severe bleeding from the wound was

prevented by the fat bodies at the base of the proleg. Using the first method, excessive bleeding may occur.

Haemocyte Identification

Fixation and Staining

For haemolymph identifications, a thin smear was made from the drop of haemolymph collected. It was then vapor-fixed in 40% acetic acid in formalin for 6 to 6 1/2 minutes. After fixation, the smear was routinely stained using Giemsa's stain, Wright's stain, Sudan Black B or Methylene Blue. The procedures for applying these stains are described in Ashhurst and Conn (4, 12).

Fresh Mounts

For fresh mount preparations, the drop of haemolymph on the microslide was covered with a coverslip immediately and ringed with petroleum jelly. Care was taken to exclude any air bubbles which would lead to melanization and darkening of the insect blood.

Phagocytic Cells

In vitro phagocytic activity was studied by putting the drop of haemolymph directly into a drop of bacterial suspension on a clean slide. The assembly was then covered with a coverslip and ringed with petroleum jelly. If air bubbles were present in the wet mount, a new slide was prepared.

For *in vivo* phagocytosis, the bacterial suspension (1 μl containing ∿1 x 10^6 cells) was injected directly into the haemolymph of the larva. Blood samples were collected every 15 minutes post-injection and examined using dark phase-contrast microscopy.

Microscopy and Photomicroscopy

The microscope used in this study was a Nikon S-Uk dark phase-contrast microscope. Photographs were taken using the same microscope equipped with a Nikon Microflex Model AFM automatic photomicrographic attachment. Cell size was measured by using a calibrated eyepiece micrometer. Information relating to photomicroscopy is described in Vetter (37).

Haemolymph Activities as Related to Infection

In this part of the study, larvae were directly injected in the haemolymph with bacterial suspensions made up in physiological saline (0.15 M NaCl). Haemolymph activities as a result of the infection were studied in two ways: First, haemocytic and phagocytic response of the larva were observed and recorded. Secondly, changes in haemolymph proteins during the course of infection were studied using acrylamide gel electrophoresis.

Bacterial Suspensions

The two bacteria used for this study were *Bacillus*

thuringiensis and *Micrococcus lysodeikticus* (13, 32). They were grown in nutrient broth at 37°C with shaking for 12 hours. Cells were collected by centrifuging at 10,000 rpm, washed twice with sterile saline solution then resuspended in saline to make a final concentration of approximately 1×10^6 *B. thuringiensis*/μl and 2×10^6 *M. lysodeikticus*/μl. These bacterial suspensions were prepared fresh each time immediately prior to injection.

Injection of Larvae

One microliter of the bacterial suspension, containing approximately 1×10^6 *B. thuringiensis* or 2×10^6 *M. lysodeikticus* was injected into the haemocoel of the larvae. Equal numbers of larvae were injected with 1 μl of sterile saline; these larvae served as controls. Injection was accomplished using a Hamilton 10 μl microsyringe. Organisms were punctured at the base of their second proleg. Before removing the needle, pressure caused by holding the larva was relieved to prevent bleeding after the needle was withdrawn.

At various hours post-injection (PI), four larvae were selected at random, punctured at the base of their proleg, and the haemolymph collected and pooled in a capillary tube precoated with phenylthiourea. The larvae were then placed back into their individual cups and observed for mortality.

Total Haemocyte, Adipohaemocyte,

and Bacterial Count

A drop of the pooled haemolymph at each hour PI was placed in a Petroff-Hausser Cell Counter for total haemocyte count (THC), adipohaemocyte and bacteria counts (see Appendix A). Also, phagocytic activity was estimated by observing 100 haemocytes randomly chosen using dark phase-contrast microscopy. The total number of bacteria phagocytized as well as the percentage of haemocytes containing bacteria was recorded. The remainder of the haemolymph was centrifuged for 10 minutes at 10,000 rpm to remove cells and then stored in the cold until all samples were collected, at which time polyacryamide gel electrophoresis was performed.

Electrophoresis

Polacrylamide gel electrophoresis of haemolymph samples was modified from the procedure utilized by Clarke (11, 28) (for details, see Appendix B). An undiluted blood sample (3.5 µl) was layered on the top of individual gel columns. Also, 1 µl of 0.2% Amino Blue Black in 50% glycerol was added onto one of the gel columns. Electrophoresis was then performed in a Glycine-TRIS buffer (pH 8.6), using a 6% gel system. A current of 2 mA/tube was found to give most satisfactory separations. Movement of the dye was monitored until it had advanced to 0.5 cm from the end of the gel. At this time the unit was shut off and the gel column removed

and placed in a screw-cap test tube. Columns were stained in 0.2% Comassie Brilliant Blue for protein. For the detailed procedure, see Appendix C.

CHAPTER III

EXPERIMENTAL RESULTS

Identification of Haemocytes

Eight categories of haemocytes were identified in the haemolymph of these corn earworm larvae. These cells occurred with different frequencies at different instars of the larvae. Each type of cell can be easily distinguished using dark phase-contrast microscopy. The following descriptions and measurements apply only to living cells under dark phase-contrast microscopy unless otherwise specified.

Prohaemocyte

Figures 1 and 2 show that these are small spherical cells with a few cytoplasmic extensions on their surface. Cytoplasm is finely granulated and has a compact dark-gray appearance. The nucleus is light-gray in color and contains numerous evenly-distributed small dark round granules which can easily be seen under oil immersion. Phohaemocytes are believed to be precursors for all other haemocytes. Size: diameter is from 5 μm to 7 μm.

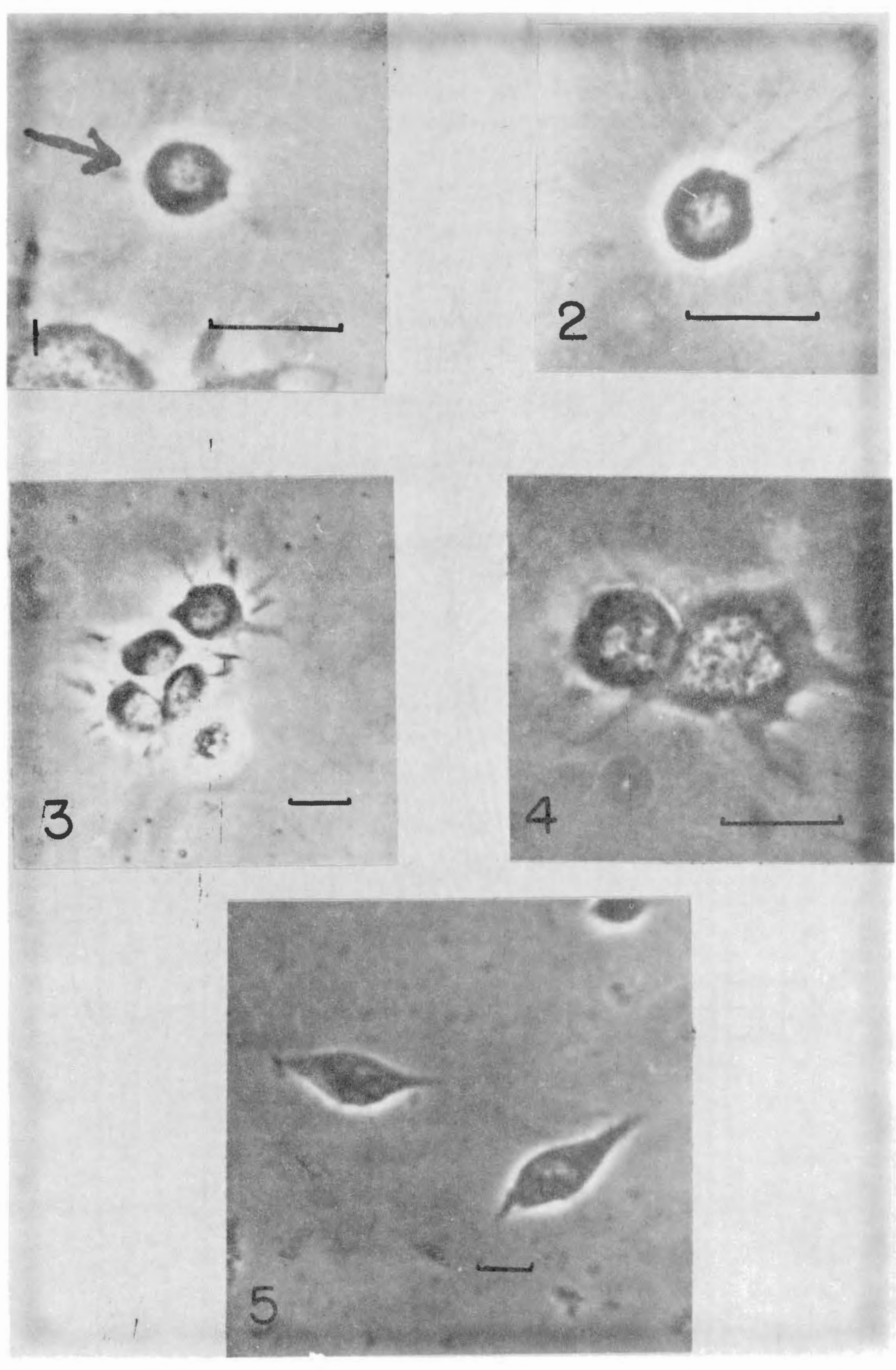
Plasmatocytes

Figures 3 and 4 show that these cells are polymorphic

Figures 1 and 2. Prohaemocytes From 3rd Instar Larva (Bar in all the figures represents 10 μm.)

Figures 3 and 4. Plasmatocytes From 3rd Instar Larva

Figure 5. Fusiform Cells From Heat Fixed Larva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with pseudopodia extending in all directions. Cytoplasm is finely granulated and appears light gray under phase-contrast microscopy. The nucleus is very similar to that observed in prohaemocytes and contains many small dark round granules; it is, however, larger and almost white in color. Size: length of cells varies from 12 µm to 15.6 µm. Average diameter of nucleus is 8 µm.

Fusiform Cells

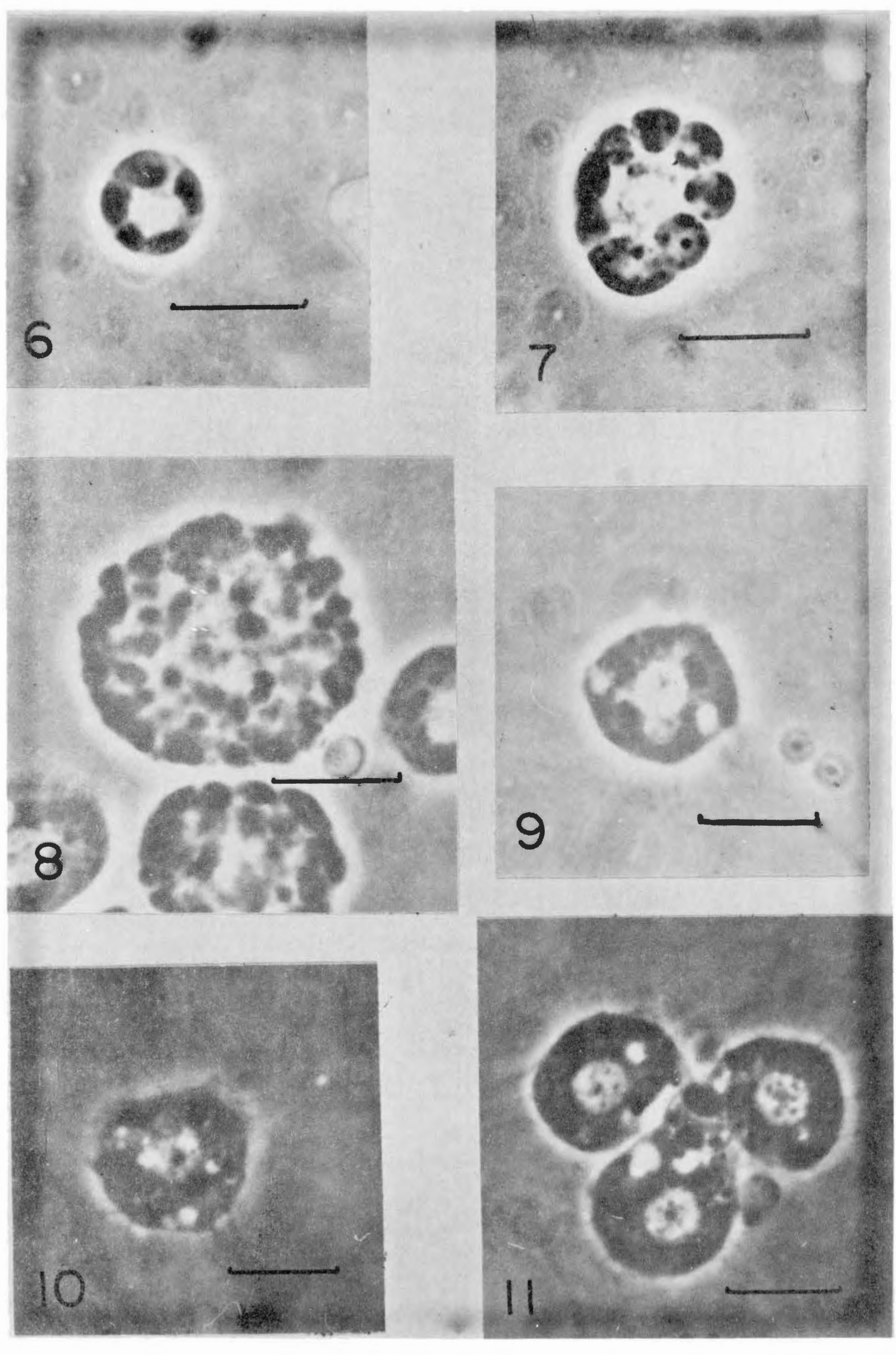
These are ovoid-shaped cells with both ends tapered into narrow points (Figure 5). The nucleus is located in the middle of the cell. Except for the shape, these cells are very similar in appearance and staining reactions to the plasmatocytes. They are not very great in number unless larvae are heat-fixed at 50°C to 60°C in water for 5 minutes prior to collection of the haemolymph. At the same time, the number of plasmatocytes decreases drastically. This leads to the belief that fusiform cells are a special form of plasmatocytes. Size: average length is about 14.5 µm. Average width is about 10.8 µm and average diameter of the nucleus is about 7.8 µm.

Spherule Cells

Figures 6 to 8 show that these cells are round and of variable size. The cytoplasm is filled with phase-dark spherules, each containing a few dark granules. The nucleus is slightly eccentric, light gray to white in color, and

Figures 6 to 8. Spherule Cells From 3rd Instar Larva

Figures 9 to 11. Adipohaemocytes From 3rd Instar Larva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usually partly obscured by the cytoplasmic inclusions. These spherules stain reddish in Giesma's Stain, indicating that they are acidic in composition. Size: average diameter is 10.4 µm; range is 8.4 µm to 14.5 µm.

Adipohaemocytes

Figures 9 to 11 show that these cells are round with very thin cytoplasmic projections extending in all directions. The cytoplasm is filled with vacuoles, granules, and fat droplets. These cytoplasmic elements are so dense that they sometimes obscure the nucleus. Size: diameter of these cells range between 8.4 µm to 12.0 µm, with an average of 9.8 µm. The nucleus has an average diameter of about 4.2 µm.

Oenocytoids

Figures 12 to 14 show that these are very large cells generally about 4 to 5 times larger than the other haemocytes. Although some are ovoid, the majority are spherical in shape. Cell surface is smooth, without any extensions. Cytoplasm is grayish and smooth, usually divided into two or more sections. The nucleus is large and extremely eccentric and is found close to the cell surface. Size: diameter is between 17 µm and 29 µm, with an average of 21.5 µm. The nucleus has an average diameter of 7.7 µm.

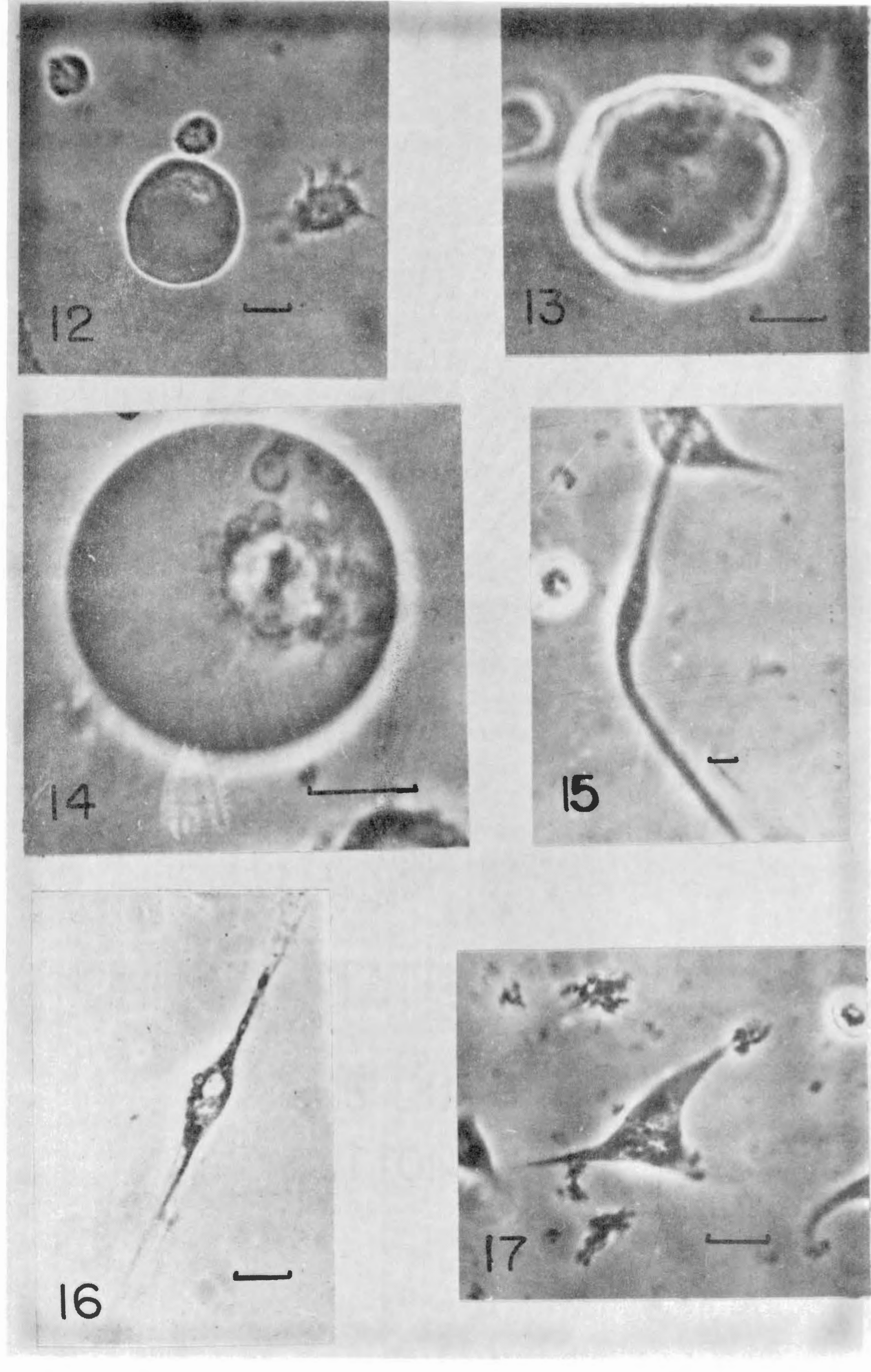
Vermiform Cells

Figures 15 and 16 show that these cells have their

Figures 12 to 14. Oenocytoids From 3rd Instar Larva

Figures 15 and 16. Vermiform Cells From 5th Instar Larva

Figure 17. Podocyte From 5th Instar Larva



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cytoplasm extended into exceedingly long arms pointed in opposite directions. The nucleus is ellipsoid in shape and situated at the middle of the very long cell. Size: cells have an average length of 61 μm (range is 42 μm to 84 μm). The average width of these cells is about 6.7 μm.

Podocytes

These cells are very rare (Figure 17). They appear only prior to pupation (5th instar). They are triangular in shape, with a whip-like extension of the cytoplasm at the apex of the triangle. The nucleus is eccentric with dark irregular-shaped granules. Since these cells do not appear very often, accurate measurements were not made. Size: approximately 20 μm from one side of the cell to the tip at the opposite side.

The eight types of haemocytes observed in the corn earworm are basically the same as the first eight classes of haemocytes detailed by Jones (19, 20). The ninth class, which he termed coagulocytes or cystocytes, was not observed, since these can be identified only by their function (coagulation of haemolymph). Comparison with observations made by others (18, 21, 23, 27, 29) reveals that similar classes of cells are present in different species of the *Lepidoptera*.

Phagocytosis

The only cells to exhibit *in vivo* phagocytic activity were adipohaemocytes. Ingestion of bacteria occurred within

15 minutes PI (Figure 18), and the number of bacteria phagocytized increased with time (Figures 19 and 20).

When phagocytosis was studied *in vitro*, adipohaemocytes failed to engulf any bacteria, even after 2 hours. Interestingly, however, the cytoplasmic extensions that occur in adipohaemocytes, plasmatocytes, and prohaemocytes could be seen to immobilize *B. thuringiensis* on their cell surface (Figures 21 and 22). This effect was also observed using *M. lysodeikticus* (Figure 23), a non-pathogenic bacterium. Lack of phagocytic activity *in vitro* has also been reported by other researchers (18, 23); however, they considered their negative results to be the result of unfavorable conditions of incubation. *In vitro* phagocytic activity by various kinds of haemocytes (adipohaemocytes, plasmatocytes, prohaemocytes, and spherule cells) has been observed by some investigators under carefully controlled conditions (1, 30, 31, 39), i.e., incubation in tissue culture media formulations.

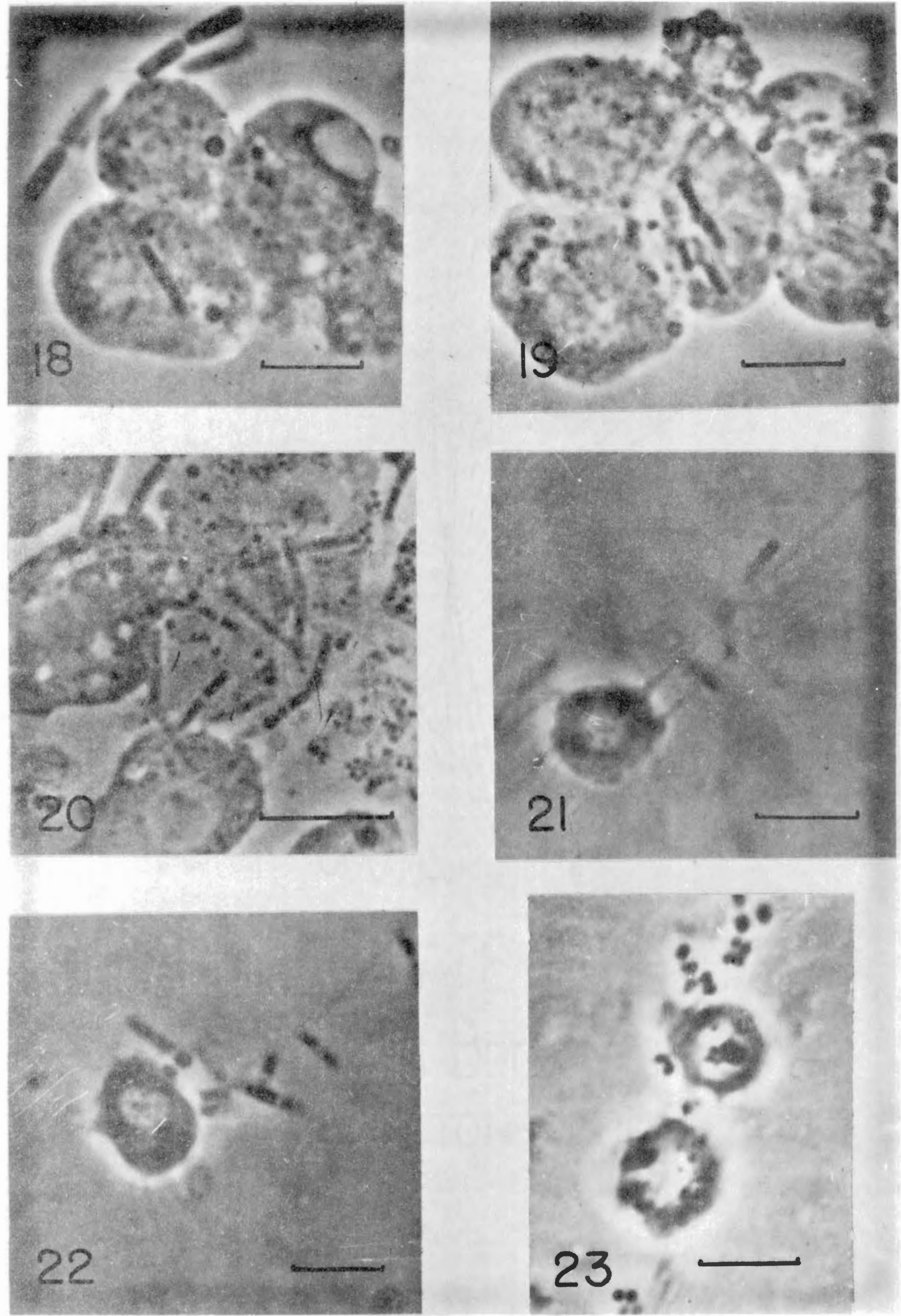
Concanavalin A, a plant lectin which specifically reacts with the carbohydrate portion of glycoproteins (15, 24, 25), was added to aid in characterization of the "sticky" surface material. Failure of Concanavalin A to inhibit the immobilization of bacteria by the haemocyte surface suggests that the responsible chemical is not a glycoprotein. Further characterization of the sticky surface material has not been attempted.

Figure 18. Phagocytosis of *Bacillus thuringinesis* by Adipohaemocytes at 15 Minutes PI

Figures 19 and 20. Phagocytosis of More Than One *Bacillus thuringiensis* Cell by a Single Adipohaemocyte

Figures 21 and 22. Immobilization of *Bacillus thuringiensis* on Cytoplasmic Extensions of Adipohaemocytes *in vitro*

Figure 23. Immobilization of *Micrococcus lysodeikticus* on Cytoplasmic Extensions of Haemocytes *in vitro*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Cellular Defense Reactions

Data relating to events that occur during the infective process with B. thuringiensis and M. lysodeikticus are given in Tables I to IV.

Tables I to III show the results of inoculating larvae with approximately 1 x 10^6 cells of B. thuringiensis (contained in 1 µl of physiological saline). The number of free cells decreased within the first 4 to 8 hours PI, after which the bacteria started to multiply and increased in number until they overwhelmed the larvae. The death rate was 7 to 8 times higher than that which occurred in the controls. Total haemocytes and adipohaemocytes increased steadily as time progressed, reaching their highest number when the bacteria were present in lowest numbers. By the time the highest death rate was reached (approximately 16 to 18 hours PI), most haemocytes had disintegrated. The majority of cells remaining (about 95%) were spherule cells.

The above changes in haemocyte number can be interpreted as a response to the presence of invading bacteria. By determining the number of phagocytized bacteria, it can also be seen that a direct relationship exists between phagocytosis by haemocytes and the number of free bacteria present in the haemolymph. A low bacterial count was usually preceded by a high number of phagocytized bacteria.

Table IV presents the results that occur after injection with approximately 1.8 x 10^6 cells of M. lysodeikticus per

TABLE I

CELLULAR RESPONSE OF CORN EARWORM LARVAE AFTER INJECTION WITH ~0.9 X 10^6 CELLS OF B. THURINGIENSIS

	HRS PI					
	0.25	2	4	8	12	16
% Larvae Dead	0	0	0	0	0	77
Bact. X 10^6/ml Haemolymph*	0.60	0.09	0.01	0.55	0.70	TNTC§
THC X 10^6/ml Haemolymph†	11.00	12.7	14.5	10.0	12.0	MD#
Adipohaemocytes X 10^6/ml Haemolymph	3.75	3.88	3.85	3.20	3.50	MD
Number of Bacteria Phagocytized¶	7	21	10	8	8	-
% Haemocytes Containing Bacteria	5	10	8	5	5	-

*Direct count of free bacterial cells in the haemolymph
†THC - Total Haemocyte Count
¶Data obtained by observing 100 haemocytes chosen at random. Usually phagocytizing cells contain more than one bacterium.
§TNTC - Too numerous to count.
#MD - Mostly disintegrated (1-5% of the haemocytes may still be intact; of these about 95% are spherule cells).

TABLE II

CELLULAR RESPONSE OF CORN EARWORM LARVAE AFTER INJECTION WITH ~1.04 X 10^6 CELLS OF B. THURINGIENSIS

	HRS PI					
	0.25	2	4	8	12	16
% Larvae Dead	0	0	0	0	80	100
Bact. X 10^6/ml Haemolymph*	0.92	0.52	0.06	5.95	93.0	TNTC§
THC X 10^6/ml Haemolymph†	7.31	4.13	16.4	6.10	4.56	MD#
Adipohaemocytes X 10^6/ml Haemolymph	2.63	3.14	6.56	3.35	MD	MD
Number of Bacteria Phagocytized¶	13	43	29	16	-	-
% Haemocytes Containing Bacteria	>10	~10	10	15	-	-

*Direct count of free bacterial cells in haemolymph.
†THC - Total Haemocyte Count
¶Data obtained by observing 100 haemocytes chosen at random. Usually phagocytizing cells contain more than one bacterium.
§TNTC - Too numerous to count
#MD - Mostly disintegrated (1-5% of the haemocytes may still be intact; of these about 95% are spherule cells).

TABLE III

CELLULAR RESPONSE OF CORN EARWORM LARVAE AFTER INJECTION WITH ~0.6 X 10^6 CELLS OF *B. THURINGIENSIS*

	HRS PI					
	0.25	2	4	8	12	16
% Larvae Dead	0	0	0	0	0	60
Bact. X 10^6/ml Haemolymph*	0.9	0.2	0.09	0.14	3.45	22.4
THC X 10^6/ml Haemolymph†	8.44	6.25	10.63	9.38	12.75	MD§
Adipohaemocytes X 10^6/ml Haemolymph	3.31	2.87	3.56	4.76	4.38	MD
Number of Bacteria Phagocytized¶	6	26	8	17	22	-
% Haemocytes Containing Bacteria	5	10	5	8	~10	-

*Direct count of free bacterial cells in the haemolymph.
†THC - Total Haemocyte Count
¶Data obtained by observing 100 haemocytes chosen at random. Usually phagocytizing cells contain more than one bacterium.
§MD - Mostly disintegrated (1-5% of the haemocytes may still be intact; of these about 95% are sphereule cells.

TABLE IV

CELLULAR RESPONSE OF CORN EARWORM LARVAE AFTER INJECTION WITH ~1.8 X 10^6 CELLS OF *M. LYSODEIKTICUS*

	HRS PI					
	0.25	2	4	6	8	12
% Larvae Dead	0	0	4	4	8	8
Bact. X 10^6/ml Haemolymph*	0.59	0.005	0.005	10.5	24.5	1.9
THC X 10^6/ml Haemolymph†	7.8	10.8	13.6	12.8	17.5	12.6
Adipohaemocytes X 10^6/ml Haemolymph	4.1	5.6	3.6	4.0	6.0	3.6

*Direct count of free bacterial cells in the haemolymph
†THC - Total Haemocyte Count

larva. The number of bacteria increased during the first 8 hours, thereafter it rapidly decreased at 12 hours PI. Prior to the decrease, an increase in the total haemocyte and adipohaemocyte count was observed. Since M. lysodeikticus is not a known pathogen, the mortality rate was expected to be low. At 24 hours PI, the death rate was less than 10%; this is very close to that which occurred in the control larvea and indicates that the death rate could be a result of the injection puncture or trauma.

Haemolymph Protein Reactions

To aid in determining possible involvement of antimicrobial haemolymph elements in the interactions described above (B. thuringiensis was found to be lysed even though they are normally not lysed by lysozyme), polyacrylamide gel electrophoresis of cell-free haemolymph at selected hours PI was performed. Representative data are presented in Figures 24, 25, and 26.

At least 13 negatively-charged protein bands can be resolved in the haemolymph of corn earworm larvae. Slight variations in the migration of these bands in the electric field, sometimes even under identical conditions, may be observed. Bands 3, 5, and 6 appear to represent the major proteins (quantitatively) in the haemolymph. Some proteinaceous materials fail to migrate more than 1 mm into the gels, thus forming a heavy staining band at the top. Fraction 3 sometimes separated into two bands; depending on the

Figure 24. Electropherograms of Haemolymph Protein From Corn Earworm Larvae Infected With *B. thuringiensis*. C = Controls; Arrows Indicate Changes in Peak Intensity and/or Peak Pattern. Left to Right: 2 Hr PI; 4 Hr PI; 4 Hr PI; 8 Hr PI; and 12 Hr PI.

Figure 25. Electropherograms of Haemolymph Protein From Corn Earworm Larvae Infected With *M. lysodeikticus*. Left to Right: Human Serum; Control at 0 Hr PI; 2 Hr PI; 4 Hr PI; 8 Hr PI; 12 Hr PI; and 16 Hr PI.

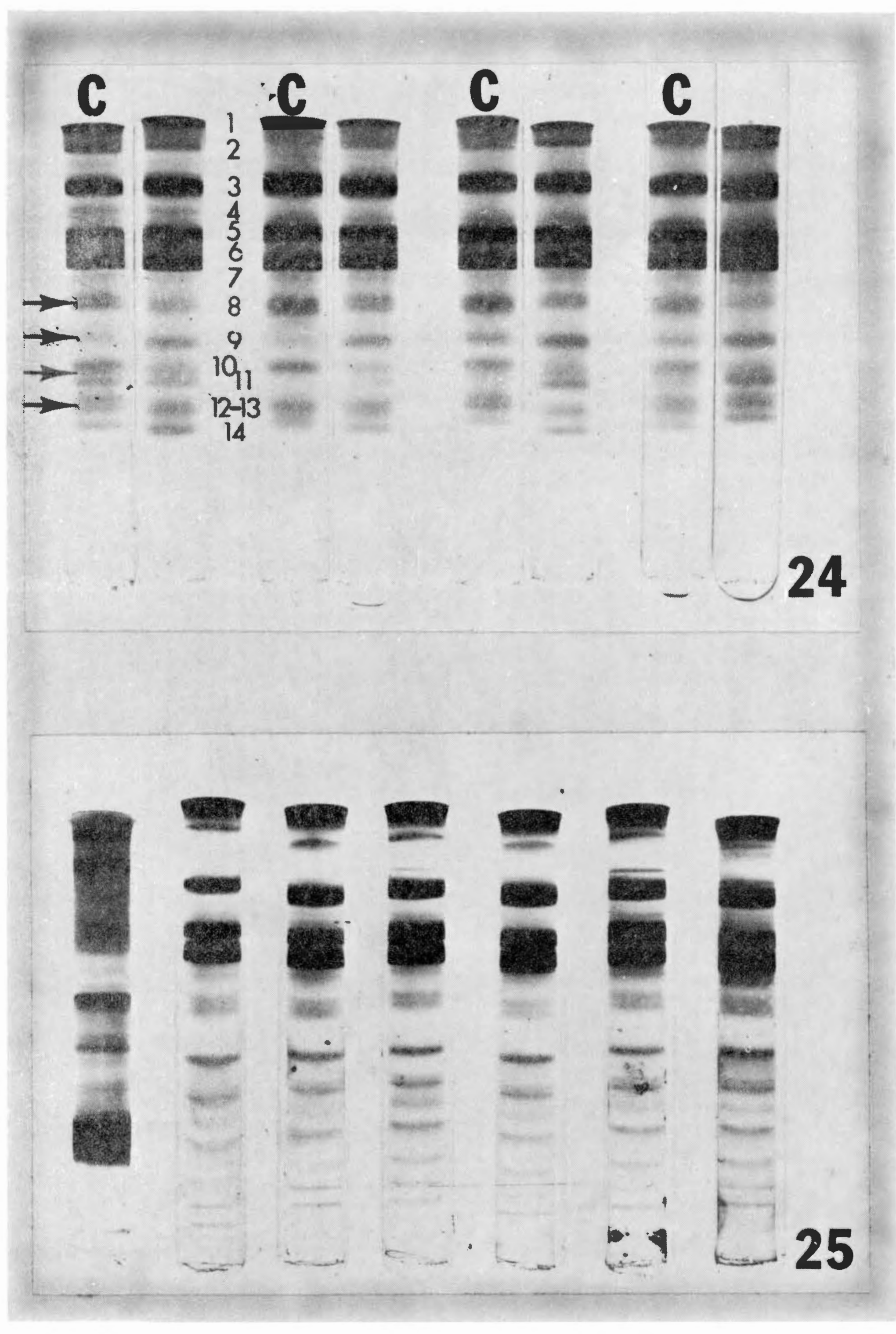
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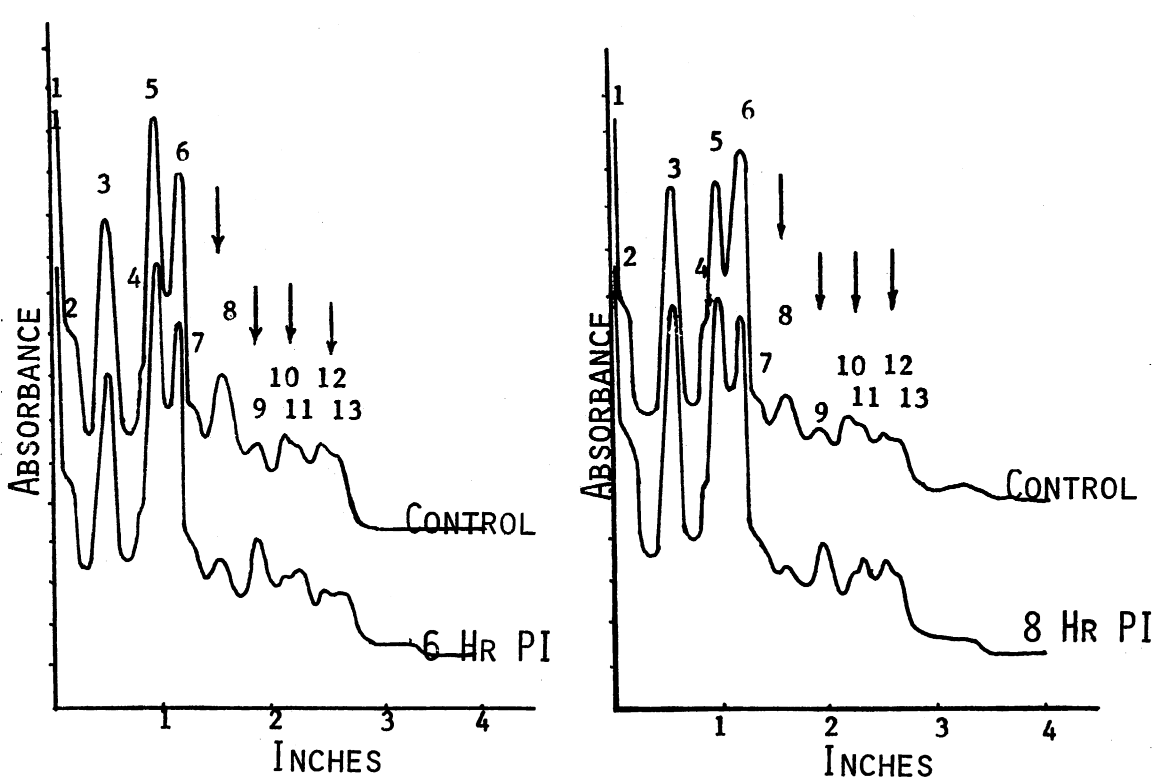
Figure 26. Densitometer Scan of Electropherograms From Corn Earworm Haemolymph Protein



ABSORBANCE
1
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CONTROL
6 HR PI
1
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INCHES
ABSORBANCE
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CONTROL
8 HR PI
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2
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INCHES

color of the haemolymph, they appear yellow and/or green before the gels are stained, indicating that they are the pigmented proteins of the haemolymph. Fraction 13, the fast-moving "front" band, follows closely behind the tracking dye (Naphtol Blue Black). Comparison with human serum reveals that fractions 7 to 13 migrate between γ-globulins and albumin; this suggests that their molecular weights probably range between 150,000 (γ-globulins) to 65,000 (albumin). Fractions 1 to 2 are composed of relatively high molecular weight proteins since they do not advance more than 1 cm into the gel.

During the course of infection by _B_. _thuringiensis_ (as early as 2 hours PI), a decrease was observed in band 8, while band 9 increased in intensity. This persisted throughout the course of infection. By 6 hours PI, band 10 starts to decrease, while band 11 increases at the same time. These changes, accompanied by other minute variations, can be seen clearly when the gels are scanned with a densitometer (Figure 26). When the larvae were injected with _M_. _lysodeikticus_ no significant qualitative nor quantitative changes in haemolymph proteins were observed (Figure 25), regardless of the dynamics of phagocytosis and removal of bacteria from the haemolymph (Table IV).

Positions of fractions 11 to 13 within the gel indicate that it is unlikely that their changes imply antibody-like reactions (agglutination); however, the presence of other haemolymph factors cannot be ruled out when compared with

other studies (5, 26, 35, 38, 43). Since all of the proteins are present in the haemolymph before the larvae are infected, changes observed indicate a response within the haemolymph to the invading microbes. These haemolymph factors may be synthesized and/or used up to bring about the removal of infecting bacteria.

The identities of these humoral factors are postulated to be bacteriolysins (such as lysozyme). These are most likely present in the haemocytes or haemolymph and would lead to the lysis of *M. lysodeikticus* or *B. thuringiensis*. Since lysozyme does not lyse *B. thuringiensis*, a bacteriolysin different from, and possibly in addition to, lysozyme must be present and active within the haemolymph.

CHAPTER IV

SUMMARY AND CONCLUSION

The best-known function of haemocytes is that of phagocytosis and encapsulation of invading organisms. In this study, eight types of haemocytes were identified in corn earworm larvae, namely: prohaemocytes, plasmatocytes, fusiform cells, spherule cells, adipohaemocytes, vermiform cells, oenocytoids, and podocytes. Of the eight types, adipohaemocytes were shown to be the primary phagocytic cells. Ingestion and immobilization of injected bacteria by these cells takes place within a very short period (15 minutes PI). Previous studies on phagocytosis have indicated that the process is a primary defense mechanism against invading microbes in insects. The ability of the insect to survive an infective dose of bacteria depends on the ability of the adipohaemocytes to remove and destroy the invading micro-organisms. This appears to also be the case for the corn earworm. The whole process appears to start with the immobilization of bacteria by sticky surface material found on the prohaemocytes, adipohaemocytes, and plasmatocytes, which eventually leads to phagocytosis by the adipohaemocytes. Bacteriolytic enzymes within the phagocytes can then proceed to destroy the bacteria. The identity of these bacteriolysins (lysozyme and

others?) are at the present unclear, but their existence is evidenced by the initial clearance of *B. thuringiensis*, final clearance of *M. lysodeikticus*, and the presence of lysed cells of *B. thuringiensis* within adipohaemocytes.

The existence of humoral factors in defense reactions was explored by the use of gel electrophoresis. At least 13 negatively charged proteins were found in the haemolymph of healthy larva. No new proteins were produced during infection, at least not in detectable amounts. Consistent changes were observed among several of these haemolymph proteins when the insects were infected with *B. thuringinesis* but not when infected with *M. lysodeikticus*. Evidence strongly suggests these changes originate within the larva as a response to the invading microbes. Specificity of these reactions toward the infective bacteria and possible relationships they may have with the phagocytic process is at this point uncertain. It appears that they are not antibody-like reactions.

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APPENDIXES

APPENDIX A

PETROFF-HAUSSER CELL COUNTER

Two types of squares were engraved on the Petroff-Hausser Counter: large and small. A drop of cell suspension was put on the counter and covered with a cover slip immediately. The number of cells in five or more small squares were counted under a microscope. Preferably the squares counted were from different areas of the counter. Concentration of the cell suspension in 10^6 cells/ml could then be calculated from the following equation:

$$\text{No. of cells} \times 10^6 = \frac{\text{Total Number of Cells Counted} \times 4 \times 10^6}{\text{Number of Squares Counted}}$$

APPENDIX B

POLYACRYLAMIDE GEL ELECTROPHORESIS

This method was modified from the system described by John T. Clarke (11). For theory and background materials concerning disc electrophoresis, one can refer to the paper of Ornstein (28).

The following stock solutions were prepared fresh every three to four weeks (depending on the frequency of usage), and kept refrigerated:

(1) Stock Solution A -

Acrylamide	15.0 gm
N,N'-methylenebisacrylamide	0.5 gm
Distilled Water	61.5 ml

(2) Stock Solution B -

Glycine	2.9 gm
Tris-hydroxymethylaminomethane (TRIS)	0.6 gm
Distilled Water	98.0 ml

(3) Stock Solution C -

N4-tetramethylene ethylene-diamine (TEMED)	2.8 μl/ml

(4) Stock Solution D -

Ammonium Persulfate (Prepared fresh each time)	1.4 μl/ml

(5) Stock Solution E -

Glycine	29.0 gm
TRIS	6.0 gm
Distilled Water	975.0 ml
(pH is adjsuted to 8.6)	

Polished Pyrex glass tubes, 7.5 cm long with an inner diameter of 8.5 mm, were used for gel column preparation.

They were kept dust-free in methanol, and were air dried before use. One end of the tubes was covered and sealed with a double layer of Parafilm. They were then stationed upright on a stand and were ready for gel layering.

Monomeric gel solution was mixed using stock solutions in the proportions shown in Table V to obtain different percentages of gels.

TABLE V

VOLUME OF STOCK SOLUTIONS FOR GELS OF DIFFERENT PERCENTAGES

Sol. A (ml)	Sol. B (ml)	Sol. C (ml)	Sol. D (ml)	Gel (%)
10.00	1.25	15.00	3.75	8.00
9.00	2.25	"	"	7.20
8.00	3.25	"	"	6.40
7.00	4.25	"	"	5.60
6.00	5.25	"	"	4.80
5.00	6.25	"	"	4.00
4.00	7.25	"	"	3.20
3.00	8.25	"	"	2.40
2.00	9.25	"	"	1.60

After the solution was mixed, it was immediately placed into the glass tubes with a syringe to the appropriate length (usually about 6 cm from the bottom). A few drops of distilled water were carefully layered on top of the gel

solution using a 100-μl microsyringe. Care must be taken to avoid disturbing the interface before the gel has hardened. Fluorescent light will enhance the speed of polymerization to within one to two hours.

Prior to electrophoresis, the Parafilm was removed, and the water layer was replaced by the electrolyte (1:10 dilution of stock solution E). Tubes were then fitted into the upper buffer-chamber. Both buffer-chambers were then filled with electrolyte. Air bubbles attaching to either ends of any column had to be removed. Haemolymph samples were directly layered on the top of each individual column. Electrophoresis was performed at a constant current of 2 mA/tube at 150 to 200 volts for 45 minutes to one hour.

After electrophoresis, the gels were removed from the glass tubes by injecting water between the gel and the glass wall with a hypodermic needle (size 20G). The gels would slip out easily when the needle was withdrawn slowly. Each gel column was then put into a screw-cap test tube for staining.

APPENDIX C

STAINING AND DESTAINING OF GEL COLUMNS

The following procedures described for staining and destaining gel columns were performed in a 60°C water bath. The method accomplishes complete destaining in half the time it normally takes to destain gels in a diffusion destainer at room temperature.

Stock Solution (destaining): 50% Methanol/7.5% Acetic Acid/42.5% Distilled Water.

Staining Solution: 0.2% Coomassie Brilliant Blue in Stock Solution.

Fixing Solution: 10% Trichloroacetic Acid (TCA).

After gels were removed from electrophoresis tubes and placed in individual screw-cap test tubes, protein bands were stained by the following steps:

(1) Fix with 10% TCA for one hour.

(2) Remove TCA and rinse 1X with water.

(3) Stain with staining solution in water bath (60°C) for two hours.

(4) Remove staining solution (can be used again; but needs to be filtered after three to four times). Rinse 1X with water.

(5) Destain with destaining solution in water bath for two hours.

(6) Remove destaining solution and add diluted (approximately 1:4) destaining solution. The gels will be completely destained within 6 to 8 hours.

(7) Destained gel columns were stored in small test tubes separately and sealed with a double layer of Parafilm.

VITA

Peter Yee-Kong Cheung

Candidate for the Degree of

Master of Science

Thesis: DEFENSE MECHANISMS OF THE CORN EARWORM, *HELIOTHIS ZEA* (BODDIE), AGAINST INVADING MICRO-ORGANISMS

Major Field: Microbiology

Biographical:

Personal Data: Born in Sawtow, China, July 17, 1950, the son of Mr. and Mrs. Cheung Chei Chwin.

Education: Attended primary school in Hong Kong; graduated from LingNan Middle School in 1970; received the Bachelor of Science degree from Oklahoma Baptist University in May, 1974; completed requirements for the Master of Science degree at Oklahoma State University on May 14, 1977.

Professional Experience: Laboratory assistant, Biology Department, Oklahoma Baptist University, 1971-1974; graduate teaching assistant, Microbiology Department, Oklahoma State University, 1975-1976; research assistant, Entomology Department, Oklahoma State University, Stillwater, Oklahoma, Summer, 1976.