EVALUATION OF ZONE ELECTROPHORESIS AND OTHER METHODS
FOR MONITORING BLOOD-SERUM PROTEINS OF THE
EUROPEAN CORN BORER DURING DISEASE

BY

Scott A. Wagner

January 20, 1982
Submitted to Dr. H. Zimmack
In Partial Fulfillment of ID 499

ABSTRACT

A quick method of determining disease in the European Corn
Borer is the final goal of my research, since a change in the tot-
al blood- serum protein concentration has been shown to indicate
disease. In studying blood- serum proteins during disease, there
are two problems: innoculation, and serum protein bioassay.

The fifth instar larvae of the European Corn Borer was used.
But even at this large size, attempts at anal injections and "spoon
feeding" of known concentrations of Bacillus thuringiensis and
Bacillus subtilis (heavy and mild pathogens, respectively) were
unsucessful. It would be ideal, for standardization purposes, to
know the amount of bacteria ingested by the larvae. Later in the
literature it was found that many researchers mix the bacteria
with the food, therefore approximating the ingested concentrations
when the larvae are small.

I have examined four bioassay methods for measuring blood-
serum protein concentrations during disease. At first, the Lowry
Protein determination method was tried. In the procedure, the
copper ion binds to long amino- acid sequences of the protein,
therefore producing color, which is read on the Spectronic 20.
Low concentrations of protein do not bind enough copper ion to
produce a color change that could be read in any spectrophotometer
available. To use this method, the blood of ten larvae must be
"pooled", which may introduce unwanted errors. Refractive index
was evaluated. Refractive index measurement of serum protein

EVALUATION OF ZONE ELECTROPHORESIS AND OTHER METHODS
FOR MONITORING BLOOD-SERUM PROTEINS OF THE
EUROPEAN CORN BORER DURING DISEASE
PART I

This work is divided into two portions. The first is an
essay on the basis of my three-year experience in researching
the European Corn Borer, <u>Ostrinia</u> <u>nubilalis</u>, and the second
portion is a scientific report on my latest research. Many
things have been tried, and consequently, many mistakes have
been made. Therefore, I include all of my thought, work, and
experiments so that the interested reader may use them to
whatever purpose he deems necessary. I also hope that this
paper will serve as a guide to the student concerned with
examining the blood (hemolymph) proteins of the European
Corn Borer. The paper is not an exhaustive study, but I
feel it contains helpful hints for anyone wishing to begin
work with this insect.

The corn borer's blood proteins are best studied at 13-
15 days post-"black head" egg stage (fifth instar). The
reason for this is simple--It is easiest to obtain tissue-free
serum samples at this age. When the larva is much younger
or smaller, one must nearly macerate it to even hope to
get 10 μl of serum after the sample is centrifuged. The

concentration is based on the principle that a higher concentration of protein will be more "cloudy" and have a higher refractive index. This method proved to be too crude for the laboratory.

Next, zone electrophoresis was tried so that blood- serum proteins could be analyzed both quantitatively (area under the curve), and qualitatively (relative width and height of peaks). Zone electrophoresis is based on the principle that proteins have different charges at a given pH, and an electrical field can be used to separate these bands on a porous cellulose acetate medium. A densitometer actually "shines light" through the resulting bands, producing peaks that correspond to the density of the bands. In the present study, control results were consistant, but larvae fed B. thuringiensis and B. subtilis showed inconsistant electrophoretic patterns. The area, width, and peaks of identical samples (experimental) varied greatly. It has been suggested that this inconsistancy might be a result of bacteria in the blood.

B. thuringiensis is known to lyse the single- layer epithelium gut cells of the European Corn Borer. As a result of this lysis, cellular enzymes are released into the blood. Martignoni and Milstead (1967), using the above principle, tested for unusual levels of glutamate- aspartate transaminase in Peridromia saucia. *Common name.* This enzyme is present in the gut cells of insects, and absent in the blood of healthy insects. Testing for this enzyme in the European Corn Borer would be far superior to the previous methods. A specific protein (the enzyme) catalyzing a specific reaction will be assayed insted of total protein, which can vary with age, sex, and circadian rhthms. The appearance of this enzyme in the blood is direct evidence of gut cell lysis.

serum is obtained by decapitating the larva in the area just behind the head and holding it perpendicular to a depression slide so that gravity drains the blood. Then the micro-hematocrit tube is placed at a 30° angle to the drop of blood (about 30 μl), and is subsequently drawn in by capillary action. This technique has proven to be effective in obtaining reason-ably pure serum as evidenced by very little white, cell con-taining debris, and somewhat consistent elecrophoretic patterns. The conventional technique of puncturing the larva behind the abdominal proleg proved to yield excess white debris and little serum. To properly conduct zone electrophoresis, about 20 μl of serum is needed (using the millipore apparatus). This is not because the serum appli-cators require such a volume, but because serum is lost in the transfer from the capillary tube either by spillage, coagulation, or evaporation. Therefore, any techniques to be used in studying the blood proteins of the European Corn Borer must require no more than 15 μl of serum. Lepidopteran hemocytes have been studied extensively (Spencer Reams, M.S. thesis; and Gupta 1971), but much less is known about corn borer blood-serum proteins. Insects are known to have much more free amino acid in the serum than vertebrates (Rockstein, 1974). It has been shown, however, that the total blood-serum protein concentration changes when an insect is diseased. (Rockstein, 1974). Many researchers have tried to relate this change to an immunological response (e.g., Baras et al., 1972), but according to Rockstein little has been confirmed.

I can say that I do not know of a single protein that has been positively identified in the European Corn Borer. I did however, show that albumin was not present when I ran a 96% pure bovine albumin standard next to a corn borer serum sample using zone electrophoresis. I found that not a single band on the serum sample corresponded to the bands produced by the albumin standard.

If one is concerned with developing screening techniques (simple physiological tests that reflect the health of the larva after it eats a pathogen), he should look for the least cumbersome test that will yield the best results (supplying important data leading to the discovery of new biological control agents). When I first began researching, I thought that the method of Lowry et al.(1951) would be ideal in determining the health of the larvae, and subsequently the usefulness of the pathogen. As I mentioned before, diseased insects are shown to have at least a change in total blood-serum protein concentration during infection, and the Lowry method had been shown to measure total blood-serum protein easily and rapidly in humans. But I could never use this method in the corn borer because I could not obtain enough serum from the larvae. One ml serum is needed for the prescribed biuret reaction to occur. When I tried to scale the reaction down stociometrically to factors of ten or one-hundred, the final volume of the reaction mixture was not readable in any spectrophotometer available.

Another problem inherent in working with such a small creature is innoculating it with the pathogen. Micro-

manipulators have been employed to, in a sense "spoon feed"
the corn borer larvae. But these attempts proved to be both
tedious and inefficient--exactly what one does not want in a
screening technique. An expert in mosquito research, Dr.
Josef Larsen, once convinced me that a bacterial suspension
could be introduced into the gut of the corn borer through
the anus and into the digestive tract. I assembled an ap-
paratus consisting of a plastic tube connected to a micro-
injector at one end, and an ultra-thin capillary tube (that
I "pulled out" using a micro-burner) at the other. Using
an unaided eye, I carefully pushed the tube through the anus
and into the digestive tract. To test the technique, trypan
blue dye was used in place of the bacterial suspension. When
the dye was injected into the gut, the larva immediately
turned blue. The dye had diffused into the hemocoel, and when
the digestive tract was examined, large perforations were
noted where either the glass tube or the pressure had torn
the gut.

Phil Eichman, a doctoral fellow in the Biology Department
at Ball State University, conducted a lengthly literature
search on the problem of pathogen innoculation. He found
that most researchers do not inject bacterial suspensions
into the anus. They use a much simpler technique of letting
the insect feed naturally. The method I adopted was that of
Van Der Geest and Wassink (1972). The larvae are placed on
medium previously mixed with known concentrations of a bacterial
suspension while in the warm liquid state. While one cannot
be sure that a larva has eaten the innoculated medium, one

can choose larvae that are seen eating or ones that are resting
in the hole they bored in selecting larvae to be tested. It
must be true that some larvae eat more than others, but at a
certain concentration of Bacillus thuringiensis (described in
the second half of the paper), for example, half of the larvae
eat a certain amount of bacteria and die. The bacterial con-
centrations given to the insect must be varied enough so that
no larva will eat more bacteria than those of the next
highest concentration. The beauty of this approach is that the
larvae are allowed to feed naturally. Injection techniques
may traumatize the larvae thus causing more cellular damage
than the pathogen itself. As the larvae begin to incur more
cellular damage, higher amounts of protein are released into
the hemolymph. The end result is that the total blood-
serum protein concentration is artificially elevated.

Source
(Martignoni
and Milstead
1967)

When I began researching blood-serum proteins as a basis
for a screening technique, it seemed that as long as a change
in total protein was shown during disease, an excellent
screening technique would be at hand. But now I am con-
vinced that the total concentration of protein in the serum,
and its electrophoretic characteristics, is so variable that
results vary greatly from day to day even when conditions
are exactly duplicated. Results obtained on the same day
through electrophoresis are even hard to interpret. There-
fore, I doubt the usefulness of this technique.

There are many practical disadvantages to the various
techniques I have explored: the Lowry method, refractometry

and electrophoresis. I will summarize them here. The Lowry method stains all protein. The copper ion binds to a protein chain no shorter than eight amino acids. Individual amino acids are not stained. This technique could work if a "micro" spectrphotometer were available. The refractometer measures the refractive index of the serum--the more protein, the higher the refractive index. But this method was originally used in the field. Better techniques are available in the laboratory. I ran over fifty samples in zone electrophoresis, and the reader is about to see some of them. The problem with electrophoresis is inconsistant patterns. The only pattern that does not seem to fluctuate is the control. Nevertheless I have found it difficult to interpret the results, as one shall see in the next portion. My suggestion is to use biochemical tests that directly show that the larva has experienced cellular damage. If successful, the glutamine oxalotransaminase test I discuss at the end of this paper would be an ideal blood test.

PART II

EXPERIMENTAL PORTION

I. Problem - Introduction

Over the past fifty years, chemical control of insects
has fallen under close scrutiny. Consequently, researchers
turned to bacteria, protozoa, viruses, etc. that are patho-
genic to certain insects. But the number of microorganisms
to be studied is overwhelming, and current methods for se-
lecting pathogens are often ineffective. For example, in over
thirty years, only two bacterial species have been employed
as effective pathogens against insects (Zimmack, 1974).
Therefore, a group of tests is needed to screen out possible
pathogens from this multitude of potential insect pathogens.

These screening tests must be highly standardized if one
intends to repeat his test frequently. Metabolic change has
been measured (Chippendale and Beck, 1966) in the European
Corn Borer, Ostinia nubilalis (Hübner) by analyzing blood
serum proteins. Chippendale and Beck used polyacrilamide gel
electrophoresis to demonstrate the variation in total blood
serum proteins from one developmental stage to another.

The most conspicuous change in the blood of a diseased
insect is hypoproteinemia (a drop in total serum protein)
(Martignoni and Mistead, 1966). Most researchers observe
a change in the total blood serum protein during the course
of insect disease. The conspicuousness of the total blood
serum protein change during disease enables the researcher to
employ simple-but-effective screening tests to survey the
pathogenicity of a given organism for the European Corn Borer.
Martignoni and Milstead, (1964) used refractometry in the

field to determine the total blood protein concentrations of
insects infected with a nucleopolyhedrosis virus. They
lauded their technique for its "simplicity and reliability."
Dr. Martignoni (personal comment) suggested that I develop
a standard test that is also simple in investigating European
Corn Borer disease in the laboratory.

II. Purpose

This study was designed to supplement my earlier paper,
"Zone Electophoretic Analysis of Total Blood Serum Proteins
to Determine Insect Disease." The new approach is that
Bacillus subtilis in addition to Bacillus thuringiensis,
var. thuringiensis were utilized to infect the insect. Also
more samples were examined as was suggested in the earlier
paper. With the new data at hand, the usefulness of zone
electrophoresis in a standard bioassay to determine insect
disease was asessed.

III. Objectives

1. To infect the European Corn Borer in the manner that
Van Der Geest and Wassink, (1972) infected Pieris brassicae.

2. To compare, both qualitatively and quantitatively,
the total blood serum proteins of fifth instar European Corn
Borers that have been infected with Baeillus thuringiensis var.
thuringiensis and Bacillus subtilis to the total blood
serum proteins of normal (control) insects.

3. To examine the usefulness of zone electrophoresis in
establishing a standard bioassay for detecting insect disease.

IV. <u>Hypothesis</u>

The total blood serum protein values for insects fed
disease-causing bacteria will differ significantly from
the total blood serum protein values of the appropriate controls.

V. <u>Materials</u> <u>and</u> <u>Methods</u>

The European Corn Borer, <u>Ostrinia</u> <u>nubilalis</u> (Hübner),
was raised on artificial food medium after Guthrie et al.
(1965). Once the medium was prepared, the agar base was
kept from solidifying by placing the medium in a $70^{\circ}c$ water
bath for the time required to complete the following operations.

25 mg. of Dipel spore powder (<u>Bacillus</u> <u>thuringiensis</u>)
was added to 10 ml. of distilled water. Five two-fold
dilutions are made, and 2 ml. of each resulting dilution was
added to 40 ml. of the liquid medium. The new mixture was
homogenized by a sterile plunger mechanism (Phil Eichman,
personal communication). 20 ml. of the liquid mixture was
poured into a sterile petri dish.

The total spore concentration of a suspension of <u>Bacillus</u>
<u>subtilis</u> was found using the "pour-plate" enumeration method.
Ten fold dilutions were made from this sample, and the concen-
tration of bacteria in per ml. of each dilution was calculated.
The media and bacteria were homogenized and poured into perti
dishes. The controls contained the medium and 1.0 ml of
sterile water.

The corn borer was transfered at the "blackhead" egg
stage to its own separate shell vial. After fourteen days of
being reared at 30 C ($^{+}_{-}1$), 70% ($^{+}_{-}4$%) humidity, and in total

darkness, the larvae were well into the fifth instar stage and had pupated by this time, many continued to eat actively. The fourteen day-old larvae have a large blood-fluid volume; therefore, a large serum sample was obtained.

Five fourteen day-old larvae were transfered (using forceps that were rinsed in chlorox) to each petri dish. The larvae ate the medium and did not seem to interfere with each other's activities. The petri dishes containing the corn borers were placed in the incubator (previous conditions) for 24 hours. After this time, a mortaility count was taken. The number of larvae that pupated was also noted.

One insect was removed (again with forceps rinsed in Chlorox to avoid medium contamination) from each dish that contained a different spore concentration. Control larvae were chosen if they had not pupated. Larvae from infected dishes were chosen on the basis of sluggishness in activity or failure to respond quickly to a stimulus, while dead and pupated larvae were avoided. Each larva was decapitated just behind the head capsule, and then was held perpendicularly so that the blood flowed onto the depression slide. The blood was drawn into a microcapillary tube. The tubes were spun in a Damon Microhematocrit Centrifuge for five minutes.

The electrophoresis cell was prepared before the experiment according to the Millipore manual (Millipore Biomedica, Acton, Massachusetts 01720) and Dr. Betty Alamong's instructions (personal communication). 5.5 ml. of barbitol buffer was placed in each well of the cell. A pre-soaked cellulose

acetate phoroslide was blotted dry and flexed upward to fit in the slots and subsequently connected the two wells. The top of the cell was secured and the cells were stored at 40°F until use.

The microcapillary tubes were broken just above the clay sealer line by pliers to eliminate the heavier white, cell-containing solid, a possible source of error (Martignoni and Milstead, 1964). The remaining straw-colored serum was deposited on parafilm, and .2 μl was carefully drawn into a Millipore wire applicator by capillary action. The applicators were held above the phoroslide until all of the samples were collected. The loaded applicators were dropped gently on the phoroslides and allowed to remain there for exactly two minutes.

The cell was connected to 100 VDC for exactly twenty-three minutes (this was found to be the optimum time). The phoroslides were removed and then immersed in Ponceu-S stain for ten minutes. Phoroslides were rinsed in three consecutive 5% acetic acid washes, with periodic shifting to aid in stain removal. The phoroslides were allowed to dry for twenty-four hours, then they were dehydrated further for five minutes in 95% ethanol. The phoroslides were then transfered to a 30/70 mixture of ethyl acetate and glacial acetic acid, which served to dissolve the cellulose acetate leaving the scarlet-stained protein bands behind. Then the phoroslides were allowed to dry for three hours.

A Gelman ultra-violet densitometer was used to demonstrate the relative densities of the migrated blood serum protein

bands. The light beam scans the dried phoroslide from the anode or positively migrating proteins, to the cathode or negatively migrating proteins (I did this as a matter of convention). The relative densities were recorded graphically, and the area under the curve was calculated in per cent of band intensities.

VI. Results

The mortality curve (see next page) was based on the per cent mortality versus spore (Bacillus thur.) concentration in μg/ml. The purpose of this curve is to relate degree of infection to concentration. Also, it may show the degree and consistancy of larvae feeding. Also, the blood of infected insects may be notably lighter than the "straw" color of control insects.

Electrophoretic patterns of the controls varied from one experiment to another; therefore, each experiment was considered individually. The curves were divided into five peaks (A, B, C, D, and E) since many of the control curves previously studied showed this pattern; however, the control in the second experiment did not. When less peaks were observed, unusually large or uncommonly arranged peaks were designated as an overlap of two peaks. The area under the various peaks was calculated by counting squares on a transparent grid. The percent of the total area under the curve for each peak was also calculated. The actual peak percentages for the first experiment are shown in table one. Mean peak values and standard deviations from experiment two are shown in table two.
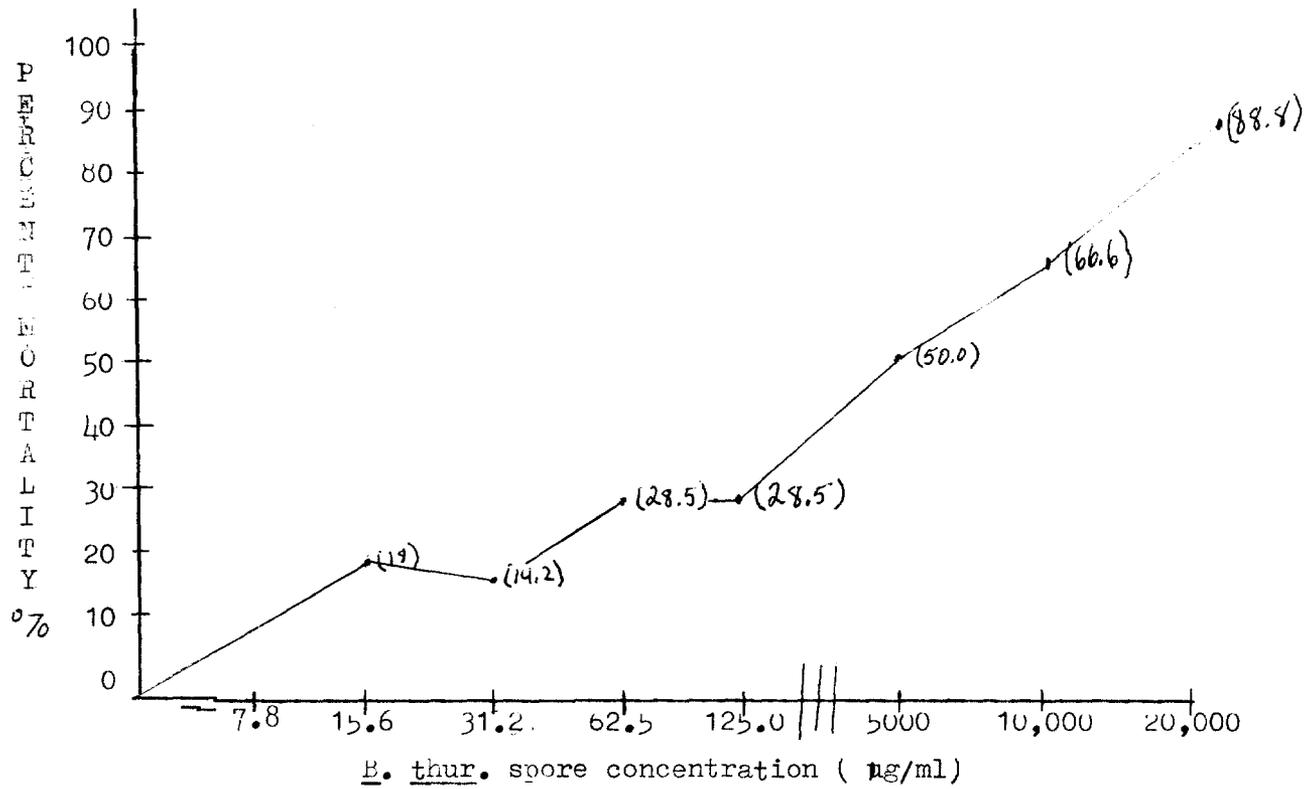
Percent mortality of fourteen day old larvae exposed to B. thuringiensis s ores for twentyfour hours.

TABLE I

ELECTROPHORETOGRAM PEAK- PERCENTAGES

PEAKS

	A	B	C	D	E
CONTROL	47.6	21.4	2.8	2.4	23.8
B. subtilis					
3.2x 10^5	10.3	43.5	31.0	7.5	7.5
6.4x 10^5	26.3	54.3	4.3	4.3	8.8
(cells/ml)					
B. thuringiensis					
5000 µg/ml	25.0	52.0	15.4	3.3	3.3
10,000 µg/ml	19.4	59.7	14.5	1.0	4.8
20,000 µg/ml	22.0	56.0	9.8	4.9	7.3

TABLE II

MEAN PEAK- PERCENTAGES AND STANDARD DEVIATIONS

PEAKS

	A	B	C	D	E
CONTROLS	16.5($\pm$20.25)	57.5($\pm$20.25)	X	21.0($\pm$1)	5.0($\pm$1
B. subtilis					
3.2x 10^5	16.5($\pm$20.25)	59.0($\pm$0.0)	X	4.0($\pm$1)	5.5(2.
6.4x 10^7	36.5($\pm$30.25)	47.5($\pm$272.25)	X	2.5($\pm$2.25)	13.0($\pm$8
12.8x 10^8	23.5($\pm$132.3)	11.0($\pm$1)	45.5($\pm$30.25)	19.0($\pm$25.0)	Y
2.46x 10^9	20.0($\pm$49)	68.0($\pm$49)	X	4.5($\pm$0.25)	8.0($\pm$1
(cells/ ml)					
B. thurigiensis					
15.6 µg/ml	22.0($\pm$25)	45.5($\pm$30.25)	23.5($\pm$0.25)	3.5($\pm$0.25)	5.5($\pm$.
32.25 µg/ml	21.0($\pm$16)	71.0($\pm$36)	X	8.0($\pm$1)	Y
62.5 µg/ml	14.5($\pm$42.25)	72.5($\pm$0.25)	X	3.0($\pm$1)	10.0($\pm$1
125.0 µg/ml	22.5($\pm$30.25)	62.5($\pm$20.25)	X	3.5($\pm$2.25)	11.5($\pm$1

X = Peaks "C" and "B" were not divisible therefore they were measured as one peak.

Y = Peak "E" was not discernible.

Evaluating experiment one qualitatively, peak "B" showed a general increase in height in larvae infected with both B. thuringiensis and B. subtilis while peak "A" showed a general decrease. Peak "C" is elevated in larvae infected with B. thur. Peak "D" shows no trends, and peak "E" decreases in both infected groups. Quantitatively, the peak-percentages back-up the decrease in the area of "A" and "E" and the increases of the area of "B". The area of "C" slighty increased in B. thur. -infected larvae, but this increase was also noted in the 3.2×10^5 cells/ml. group of B. subtilis. The area under a peak is not totally conclusive--the sharpness or height of a peak shows that more protein has been confined to a smaller band; therefore, they have very simular charges. A wider band produces a rounder peak. This means that the proteins are less alike electrically.

In experiment two, no qualitative trends were seen in peak "A". Peaks "B" or "B+C" showed an increase in the B. thur. group but a decrease in some B. subtilis groups. Peak "E" fluctuated in the B. subtilis group while a general increase was seen in the B. thur. group. Peak "D" decreased in both groups. Quantitatively, the standard deviations are great. Most of the disagreement could be attributed to the crude method of counting squares used to find the peak-percentages. Nevertheless, an evaluation will be made. Peak "A" slightly increases for both groups (as does peak "B" or "B+C") over the control values. Peak "D" decreased in both experimental groups while "E" increased slightly.

VII. Discussion

The LC$_{50}$ seems to fall between 5000 and 125 µg/ml of B. thuringiensis. This contradicts an earlier finding of about 50 µg/ml for the LC$_{50}$. No deaths were observed from B. subtilis; however, the larvae were notably sluggish to sensory stimulation after having been infected.

The electrophoresis results are too variable to assemble any standard values for control or infected larvae. The experimental curves varied from control curves. Duplicate control durves in each individual experiment were amazingly simular. Duplicate infected curves were usually more deviant. This may be a result of one larva ingesting more bacteria than the others.

Electrphoresis of European Corn Borer blood serum proteins is interesting to study, but I think that it would be a poor screening technique. Better equipment might help; however, the real problem lies in the approach of analyzing all serum proteins. The overall "picture" is too variable. Future research should be concerned with finding specific proteins that are direclty produced from the desease. Martignoni and Milstead (1962), tested the blood of Peridromia saucia (which had been infected with viruses) for glutamate-Asparatate (or oxaloacetate) transaminase activity. This enzyme is shown not to be present in the blood of healthy insects but is present in insects that are diseased with organisms that cause cell lysis. I plan to test for this enzyme in the European Corn Borer when infected with B. thuringiensis, a cell-lysing pathogen.

If this enzyme is found in infected larvae, an excellent screening test could be developed. The reagents to test for this enzyme are readily available (standard SGOT test kit). The test is specific for GOT, the reaction only requires a few minutes, and the results can be read on a spectrophotometer.

References Cited

Allamong, Betty; Bio. 411/555 Lab #2 (personal communication)

Chippendale, G.M., and S.D. Beck. 1966. Haemolymph proteins of Ostrinia nubilalis during diapause and prepupal differentiation. J. Insect Physio. 12:1629-38.

Gupta, A.P. Insect Hemocytes. Cambridge Press; New York, 1979.

Guthrie, W.D., E.S. Raun, F.F. Dicke, G.R. Pesho and S.W. Carter, 1965. "Laboratory production of European Corn Borer Egg Masses." Iowa St. J. Sci. 40:65.

Lowry, O.H., N.J. Rosbrough, A.L. Farr, and R.J. Randall. "Protein measurement with folin-phenol reagent." J. Biol. Chem. 193:265, 1951.

Martignoni, M.E., and J.E. Milstead, "Hypoproteinemia in a noctuid larva during the course of a granulosis." J. Invert. Path., 8:261, 1966.

Martignoni, M.E., J.E. Milstead, "Hypoproteinemia in a noctuid larva during the course of a granulosis." J. Insect. Path. 6:517, 1964.

Rockstein, M. The Physiology of Insecta, vol. 5. Academic Press; New York, 1974

Van Der Geest, L.P.S., H.J.M. Wassink. "Standardization of Bacillus thuringiensis preparations: A new bioassay method with Pieris brassicae as a test insect." J. of Inv. Pathology,

Vinson, S.B., W.J. Lewis, "Electrophoretic study of protein changes between several developmental stages of three species of Heliothis." Comp. Biochem. Physiol., 28:215.

Zimmack, H.L., "What is the future of biological control of insects?" Proc. North Central Branch, Ent. Soc. Amer., 22:103, 1974.