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 total blood-serum protein concentration has been shown to indicate disease. In studying blood-serum proteins during disease, there are two problems: inoculation, 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 unsuccessful. 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, *Ostrinia nubilalis*, 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 consistent, but larvae fed *B. thuringiensis* and *B. subtilis* showed inconsistent electrophoretic patterns. The area, width, and peaks of identical samples (experimental) varied greatly. It has been suggested that this inconsistency 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-succinate transaminase in *Peridroma saucia*.

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 instead of total protein, which can vary with age, sex, and circadian rhythms. 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 microhematocrit 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 reasonably pure serum as evidenced by very little white, cell containing debris, and somewhat consistent electrophoretic 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 applicators 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 hemocytoblasts 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 stoichiometrically 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 apparatus 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 lengthy literature search on the problem of pathogen inoculation. 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 inoculated 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 concentrations 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.

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 convinced 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. Therefore, 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" spectrophotometer 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 inconsistent 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 pathogenic to certain insects. But the number of microorganisms to be studied is overwhelming, and current methods for selecting 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, *Ostina 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) (Martironi and Mistlead, 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. Martironi and Mistlead, (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. Martignon (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 Electrophoretic 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 assessed.

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. Hypothesis

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. Materials and Methods

The European Corn Borer, Ostrinia nubilalis (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°C water bath for the time required to complete the following operations.

25 mg. of Dipel spore powder (Bacillus thuringiensis) 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 Bacillus subtilis was found using the "pour-plate" enumeration method. Ten fold dilutions were made from this sample, and the concentration of bacteria in per ml of each dilution was calculated. The media and bacteria were homogenized and poured into petri dishes. The controls contained the medium and 1.0 ml of sterile water.

The corn borer was transferred at the "blackhead" egg stage to its own separate shell vial. After fourteen days of being reared at 30°C (±1°C), 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 mortality 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 Wilstead, 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 fonceu-3 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 transferred 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 scars 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 consistency 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. thurigiensis* spores for twenty-four hours.
TABLE I
ELECTROPHORETIC PEAK - PERCENTAGES

<table>
<thead>
<tr>
<th>PEAKS</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>47.6</td>
<td>21.4</td>
<td>2.8</td>
<td>2.4</td>
<td>23.8</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>3.2 x 10⁹</td>
<td>10.3</td>
<td>43.5</td>
<td>31.0</td>
<td>7.5</td>
</tr>
<tr>
<td>(cells/ml)</td>
<td>6.4 x 10⁵</td>
<td>28.3</td>
<td>54.3</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>B. thuriniensis</strong></td>
<td>5000 pg/ml</td>
<td>25.0</td>
<td>52.0</td>
<td>15.4</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>10,000 pg/ml</td>
<td>19.4</td>
<td>59.7</td>
<td>14.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>20,000 pg/ml</td>
<td>22.0</td>
<td>56.0</td>
<td>9.8</td>
<td>4.9</td>
</tr>
</tbody>
</table>

TABLE II
MEAN PEAK - PERCENTAGES AND STANDARD DEVIATIONS

<table>
<thead>
<tr>
<th>PEAKS</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL OLS</td>
<td>16.5(+20.25)</td>
<td>57.5(+20.25)</td>
<td>x</td>
<td>21.0(+1)</td>
<td>5.0(+1)</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>3.2 x 10⁹</td>
<td>16.5(+20.25)</td>
<td>59.0(+20.25)</td>
<td>x</td>
<td>4.0(+1)</td>
</tr>
<tr>
<td>(cells/ml)</td>
<td>6.4 x 10⁵</td>
<td>36.5(+30.25)</td>
<td>47.5(+22.25)</td>
<td>x</td>
<td>2.5(+2.25)</td>
</tr>
<tr>
<td></td>
<td>12.8 x 10⁵</td>
<td>23.5(+12.25)</td>
<td>11.0(+1)</td>
<td>45.5(+30.25)</td>
<td>19.0(+25.0)</td>
</tr>
<tr>
<td></td>
<td>2.46 x 10⁷</td>
<td>20.0(-49)</td>
<td>68.0(-49)</td>
<td>x</td>
<td>4.5(+0.25)</td>
</tr>
<tr>
<td><strong>B. thuriniensis</strong></td>
<td>15.6 pg/ml</td>
<td>22.6(+25)</td>
<td>45.5(+30.25)</td>
<td>23.5(-0.25)</td>
<td>3.5(+0.25)</td>
</tr>
<tr>
<td></td>
<td>32.25 pg/ml</td>
<td>21.0(-16)</td>
<td>71.0(-36)</td>
<td>x</td>
<td>8.0(+1)</td>
</tr>
<tr>
<td></td>
<td>62.5 pg/ml</td>
<td>14.5(+42.25)</td>
<td>72.5(+0.25)</td>
<td>x</td>
<td>3.0(+1)</td>
</tr>
<tr>
<td></td>
<td>125.0 pg/ml</td>
<td>22.5(+30.25)</td>
<td>62.5(+20.25)</td>
<td>x</td>
<td>3.5(+.25)</td>
</tr>
</tbody>
</table>

X = Peaks "C" and "E" 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 "E". The area of "C" slightly increased in *B. thur.*-infected larvae, but this increase was also noted in the $3.2 \times 10^5$ 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 similar 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 "D+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 LC₅₀ seems to fall between 5000 and 125 μg/ml of B. thuringiensis. This contradicts an earlier finding of about 50 μg/ml for the LC₅₀. 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 curves in each individual experiment were amazingly similar. Duplicate infected curves were usually more deviant. This may be a result of one larva ingesting more bacteria than the others.

Electrophoresis 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 directly produced from the disease. Martignoni and Milstead (1967) tested the blood of Feridromia saucia (which had been infected with viruses) for glutamate-Aspartate (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

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


