THE STIMULATORY EFFECT OF VARIOUS FOODS ON PROTEOLYTIC DIGESTION IN ADULT Aedes aegypti Linn.

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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Approved by:

Adviser
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Special thanks is due Dr. Edward H. Bohl of the Department of Bacteriology for his assistance in securing sheep blood necessary for this work. The plasma protein fractions used in this work was provided by the Research Laboratories of Armour and Company.

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INTRODUCTION

Over three thousand years ago a Chinese scholar wrote of "paroxysmal manifestations of chills and fever" which are construed to be symptoms of malaria. Malaria was recognized as a disease in ancient Hindu writings in the Atharva Veda. Many Greek cities declined in importance as malaria became endemic. Later Lower Italy, previously heavily colonized, became infected with malaria. As the Roman Empire instilled building reforms, the swamps and marshes were drained, reducing the incidence of malaria around Rome. During the Middle Ages, however, several armies attacking Rome were defeated by the "Roman Fever" (Hispan, 1944).

Alphonse Laveran first discovered the malarial parasite in a patient's blood in 1880. In 1895 Sir Ronald Ross described malarial parasites in the midgut of mosquitoes but could not identify the changes in them. By 1898 he was able to demonstrate the path of the malarial parasites through the mosquito vectors. This malarial mosquito theory had been proposed by A. T. A. King, an American surgeon, in 1893 and was confirmed by Craszi and others in 1898.

Since that time, much money and time have been devoted to the study of malaria and its vectors. However, most of the research and financial support for it has gone into the fields of intermediate host symptomatology and treatment; mosquito classification, bionomics, and control; and taxonomy, morphology, and life cycle of the malarial parasites. There is a paucity of information on the physiology of the various parasites, especially pertaining to their infectivity of mosquitoes. According to Huff (1941 a), "Hardly any other phase of
malariology has been so badly neglected as the study of all factors influencing infection in the mosquito."

Of the relatively small amount of data dealing with the infectivity in mosquitoes, a large part of it deals with studies of strains of mosquitoes resistant or susceptible to strains of malarial parasites. Huff (1941 a) concluded that, other than the genetic characters responsible for differences in susceptibility, temperature greatly influences the infectivity and growth of oocysts in the mosquito. Hovanitz (1947) states that infectivity variation between species must be hereditary, whereas individual variation to infection is non-hereditary, but environmental. Although his data support the idea that the quantity of blood ingested plays only a secondary role in the degree of infectivity, he suggests "...an agent influencing development of cysts in some mosquitoes which also influences development of eggs in the same mosquitoes. It is not known what this agent may be, but it is probably connected with the processes of digestion and absorption."

Huff (1934) found no difference between digestion rates in susceptible and resistant strains of mosquitoes. Eyles (1952 b,c) studied the various plasma proteins and intrinsic factors connected with the physiological state of the vertebrate host which influence mosquito infection.

This particular study was undertaken to extend the small field of knowledge of the effect of various foods on digestive enzyme stimulation. Since the work of Schlottke (1937 a, b,c), there has been only the studies of Day and Rowning (1949), Fisk (1950), and Fisk and Shambaugh (1952) on the stimulatory effect of food on
enzyme elaboration. Such research serves to fill in many gaps in the knowledge of the physiology of adult mosquitoes, a physiology not well understood yet extremely important in all considerations of malarial infectivity in mosquitoes.
METHODS

Aedes aegypti Linn., were selected for this study because of the relative ease with which great numbers can be reared. They were obtained from the Public Health Service Project in the Department of Zoology and Entomology which maintains an active colony for its own use.

Female mosquitoes four to six days after emergence were transferred to a lamp chimney covered at the top with cheesecloth. Four-to-six-day-old mosquitoes were used, for they feed most readily at that age and by that time have disposed of any larval food which remained in the digestive tract at emergence (Fisk, 1950).

Feeding and Dissection Techniques

Mosquitoes were fed either from the bare forearm placed on the cheesecloth covering the top of the lamp chimney containing them or through an artificial membrane in a heated feeding apparatus to be described later. The mosquitoes were attracted to the arm of warmed membrane and fed through the gauze. After each one had engorged partially with blood or a substitute solution, she was withdrawn from below with an aspirator which was inserted into the chimney through an opening in cross-slitted rubber dental dam stretched over an embroidery hoop. Each female was placed in a separate vial which was stoppered with a wad of dampened cotton. The fed mosquitoes were placed in an incubator at 30°C.

After six or eighteen hours for digestion, the midguts were dissected from the mosquitoes. Each mosquito was momentarily stunned by a sharp rap of the vial against the palm of the hand. She was decapitated on a paraffin-covered single concavity slide under a
binocular dissecting microscope. The tip of the abdomen, approxi-
mately the last two segments, was removed with the aid of jeweler's
forceps and a Hagedorn needle. There was a drop of Levy's
mosquito saline in the depression of the slide. The needle was
thrust into the thorax of the mosquito to hold her. The mosquito
was placed in the saline to facilitate dissection and to prevent
desiccation and undue contamination by scales. The abdomen was
torn with the forceps at the proximal end of the abdomen, near the
thorax. By grasping the wall of the abdomen at the distal end,
it could be removed quickly, leaving most of the internal viscera
immersed in saline. The hind-gut, malpighian tubules, diverticula,
and fore-gut were removed. The midguts were accumulated in small
shell vials which contained a drop of chilled saline. The vial
was placed on ice during dissection and was stored in a deep
freezer at a temperature of -15°C as soon as dissection was
complete.

Individual Mosquito Weighing

In this laboratory, as similarly reported by Roy (1936), the
range of weights of mosquitoes at a constant age is greater than the
average weight for an individual. In order to determine accurately
the amount of blood ingested by a female mosquito during feeding, it
was necessary to obtain individual weights.

1- A Hagedorn needle is a common dissecting needle which has been honed
to form a triangular tip. This may serve as a microscalpel, probe,
or retractor.
2- The formula for Levy's mosquito saline is 7.5 grams of sodium
hydroxide, 0.62 gram of potassium chloride, 0.40 gram of calcium chloride
per liter; then add one milliliter of 0.1 normal potassium hydroxide
to bring the solution to pH 7.0.
A small, rectangular, plastic cage was constructed (3.6 cubic centimeters) to hold the mosquitoes while weighing them on a Roller-Smith Precision Balance (spring torsion). The mosquitoes were anesthetized with carbon dioxide before weighing. With an aspirator they were placed in marked shell vials and were allowed to feed on the bare forearm. The amount of blood fed was regulated by disturbing the insect when feeding. Each mosquito was reweighed soon after feeding in the same manner. The vials were stoppered with a wad of damp cotton and were placed in an incubator at 30°C. for eighteen hours before dissection.

Artificial Feeding Apparatus

It was necessary to construct an apparatus to keep artificial blood solutions at a constant temperature (10°C.). An "oven" was constructed as follows. Two sizes of Lucite plastic tubing were used: 1) outside diameter, 1.5 cm.; inside diameter, 3.5 cm.; length 3 cm. and 2) outside diameter, 5 cm.; inside diameter, 1.14 cm.; length 5 cm. The edges of the tubing were ground flat and smooth with alumina and powdered carborundum. The former smaller tube was covered at one end with Baudruche Capping Membrane held in place with a rubber band. This acted as a reservoir for the solutions. Nichrome wire was wound around the outside of the larger tube (eight times). Regular 110 volt copper extension wire was soldered to the nichrome wire. The wires were held in place with an outer insulating layer of furnace cement about one centimeter thick. The smaller tube containing the solutions was placed within the larger tube. This apparatus is a modification of that described by Greenberg (1949).
A cover was prepared for the larger tube to prevent excessive heat loss. A thin, circular, plastic disc 6.3 cm. in diameter was joined with acetone to a plastic ring 4.3 cm. in diameter and 1.5 cm. wide. The heating element was joined in series with a variable rheostat and a 120 volt, 100 watt incandescent bulb. It was necessary to vary the rheostat manually since there was no thermostat in the circuit.

The assembled feeding apparatus was placed on the cheesecloth covering the top of the lamp chimney containing mosquitoes. The capping membrane of the inner tube, wetted with distilled water, rested on the cheesecloth. Accurate readings of the temperature at the membrane surface were taken with a Leeds and Northrup single range potentiometer indicator connected to a thermocouple of copper and constantan placed between the cheesecloth and the capping membrane.

Before deciding to use the Baudruche Membrane for capping the smaller tube, it was tested against a natural animal mesentery. Large glass tubes were covered at one end with each of the membranes. Mosquitoes were introduced into the tubes which were stoppered with cotton. Membranes were moistened and placed against the bare forearm to induce feeding through them. Feeding occurred most readily through the Baudruche Membrane. Yet difficulty was encountered in being able to secure large numbers of mosquitoes to feed artificially with the apparatus. Various temperature gradients with the atmosphere were tried. Human perspiration did not seem to attract them effectively under such conditions. The membranes were treated three hours
with a solution of pepsin and later with absolute ethyl alcohol. Neither treatment increased the rate of feeding.

**Preparation of Blood Substitute Solutions**

In *Aedes aegypti*, ingested blood is taken directly into the midgut or rarely into the diverticula. Sugar solutions without erythrocytes, in practically all instances, were taken into the diverticula (Trembley, 1952). Most protease activity occurs in the midgut after feeding (Fisk, 1950; Fisk and Shambaugh, 1952). In view of these facts and the technique described by Greenberg (1919), it was decided to use sheep erythrocytes with substitute solutions so that the solutions would be primarily dispatched to the midgut. Fresh sheep blood was defibrinated by mixing with one-tenth of its volume with three percent sodium citrate. Whole sheep blood was centrifuged ten minutes at 5500 rpm. The supernatant serum was siphoned off. The remaining erythrocytes were diluted to the original volume with physiological saline (0.85% sodium chloride) and stirred before centrifuging. The washing procedure was repeated twice more. The subsequent thrice-washed erythrocytes were used in all tests requiring erythrocytes.

It was decided to test for any response in protease activity to various fractions of whole blood and to plasma proteins. The following solutions were offered: whole human blood, whole sheep blood, washed erythrocytes (cells) / saline, erythrocytes / serum dialyze, erythrocytes / serum proteins, erythrocytes / 0.6% bovine fibrinogen, erythrocytes / bovine albumin, erythrocytes / bovine gamma-globulin, erythrocytes / gum acacia. All of these mixtures were used in a 1:1 volume ratio, except the solution of
erythrocytes / fibrinogen / albumin / gamma-globulin that was in the ratio of 3:1:1:1.

Several techniques were tried to obtain a protein-free plasma. Trichloroacetic acid treatment ended in a useless precipitation. Heat coagulation and filtration through sintered glass equally were unsuccessful. Tungstic acid precipitation produced a usable solution. However, after a mosquito had fed on such a solution, the excretion rate increased greatly and the individual died if she was not able to void most of the meal within a couple of hours. Mortality is thought to be due to the toxicity of the tungstate radical. The technique used successfully was dialysis. Ten ml. of sheep blood was placed in Visking tubing. It was dialyzed into 30 ml. phosphate buffer, pH 7.8, for three hours in an Aminco Mechanical Dialyzer. The dialyzate gave a negative test for proteins upon the addition of trichloroacetic acid. Plasma protein fractions were dissolved in distilled water. The solution of fibrinogen contained between 40 to 50 percent sodium citrate to prevent its coagulation upon the addition of erythrocytes.

Injection Technique

A series of injection tests was run. A group of mosquitoes was allowed to feed on the bare forearm. They were allowed to digest the meal eighteen hours. The technique used for injection has been described recently by Shambaugh (1952). Before dissection of the fed mosquitoes, a small amount of mosquito saline was injected into the hemocoele with the mouth syringe to increase the blood volume. Dissection proceeded as described above. The mixture
of insect blood and saline was injected into unfed mosquitoes. In four instances the midgut contents were mixed with saline and injected into unfed mosquitoes. Dissections of injected mosquitoes were made eight to ten hours later.

Enzyme Analysis

Chromophoric protein derivatives are formed by the coupling of diazotized aryl amines with proteins in an alkaline solution. Such azoproteins are completely precipitated by trichloroacetic acid yielding a colorless filtrate. Digestion of a solution of such proteins by proteolytic enzymes results in the formation of colored components soluble in trichloroacetic acid (Charney and Tomarelli, 1947). The appearance of color in the trichloroacetic acid filtrate depends on an intact diazotized amino acid or peptide fragment. The intensity of color in the filtrate following protease action is a direct function of the proteolytic enzyme activity and its measurement (as optical density) serves as the basis of this technique. Since the color concentration relationship of azocasein and its digestion products obey Beer's Law, optical density may be substituted for protein concentration, thus eliminating the necessity for nomographs.

The substrate used was prepared as follows. Sulfanilamide-azocasein substrate stock solution containing 25 mg. of azocasein and five mg. of sodium bicarbonate per milliliter was prepared by dissolving 2.5 gms. of azocasein in 50 ml of 1.0% sodium bicarbonate at 60° C. with stirring. The pH was adjusted to 8.3 and the solution was diluted to 100 ml with distilled water. This stock solution was stored at 5° C.

The procedure for the analysis was as follows. The dissected-
out midguts were removed from the deep freezer and homogenized without an abrasive. One and one-half ml. of Clark and Lubs standard phosphate buffer, pH 7.8 (Morrow and Sandstrom, 1935), were added to the enzyme brei. Enough saline was added to bring the total volume of the solution to 3.5 ml. The solution was stirred. Three ml. were pipetted into six micro test tubes, 0.5 ml. per tube. Two such tubes were placed in a boiling water bath for twenty minutes while the other four were put into the deep freezer. After twenty minutes the boiled blanks were placed in the deep freezer with the others until they were approximately the same temperature (five minutes). The series was removed from the freezer and 0.25 ml. of azocasein substrate was added to each tube. The mixture was stirred again and placed in a constant temperature water bath at 40°C ± 0.5°C for incubation which lasted five hours. The incubated runs and blanks were removed from the water bath and poured into six numbered centrifuge tubes, each containing 1.5 ml. of ten percent trichloroacetic acid. Each incubation tube was rinsed into the centrifuge tube with one milliliter of acid. The precipitate was separated by centrifuging, ten minutes in a Sorvall Angle Centrifuge at 5500 rpm. Two and one-half ml. of 0.5 normal sodium hydroxide was added to 2.5 ml. of supernatant in a calibrated Klett colorimeter tube to intensify the color of the supernatant. The optical densities were determined by placing the solutions in a Klett-Summerson photoelectric colorimeter using a 440 mp blue filter. The technique was checked using 0.1%, 0.01%,
and 0.001 M solutions of powdered trypsin.

The above procedure was used for protease determinations of dissected midguts in lots of twenty or ten. A modified technique was used for less than ten midguts in a dissection. In it a boiled blank and three replicate runs were used. One milliliter of buffer is added to the homogenate of the midguts and the total is brought up to two milliliters with mosquito saline. The remainder of the technique is as before.

All glassware coming into contact with enzyme material and substrate was cleaned with concentrated nitric acid, rinsed three times in tap water, and rinsed four times in distilled water. All other glass material was washed in water containing a commercial trisodium washing powder (Calgonite), rinsed several times in tap water, and rinsed four times in distilled water. The standard acid-dichromate cleaning solution was avoided since it affects the enzyme actions adversely.
RESULTS

The conclusions of Fisk and Shambaug (1952) on the stimulation of proteolytic digestive enzymes after a blood meal as opposed to a meal on a sugar solution serve as a basis for further studies of the effect of various blood fractions on protease elaboration in the midgut. The amount or condition of the blood meal within the midgut at the time of dissection have no effect on this technique, for the colored materials would be equal in both blanks and runs while the blood proteins would be removed by the trichloroacetic acid precipitation. The amount of protease activity determined was the amount of available protease at the time of dissection, minus losses due to technique.

In the following tables and figures, protease activity will be expressed in terms of optical density of the final solution described in the previous section. Optical density is a direct function of protease activity, being derived from the readings of the colorimeter by multiplying them by the constant 0.002 (Hawk, Oser, and Summerson, 1947). In these tables the number of midguts in the final solution read in the colorimeter depends on the initial number dissected and the technique followed. These data will be discussed later and are found in Table IV.

Results with Various Blood Fractions

Data of preliminary tests of the effect of various fractions of whole blood digested by mosquitoes for six hours appear in Table I. Starved, or unfed individuals, were used as a check. Thrice-washed erythrocytes were suspended in physiological saline to find the enhancement in enzyme activity due to the erythrocytes in an inert
medium. There was a slight increase in activity due to the addition of the cellular proteins. The addition of the prepared non-proteinaceous serum dialyzate increased the protease activity one and one-half times. This relationship will be recalled later. Both whole human blood from the forearm and whole sheep blood fed artificially were tested.

whole human blood induced a slightly higher activity than sheep blood. These relationships may be seen in Figure 1.

Since the peak of digestive enzyme activity was shown to be eighteen hours after feeding (Shanbaugh, 1951), it was decided to run the next series of experiments under those conditions (see Table II). The same ratio exists between the protease activity of mosquitoes fed serum dialyzate and those fed erythrocytes in saline as in the previous series of experiments. Gum acacia was fed to discover any stimulatory effect due to viscosity or midgut distention. There was none.

Three bovine plasma protein fractions (albumin, fibrinogen, and gamma-globulin) were fed separately in 0.6% solutions. Protease activity with fibrinogen was not different from that with serum dialyzate. Activity with albumin was slightly higher, whereas gamma-globulin increased enzyme activity substantially. When non-dialyzable sheep serum proteins were fed, a significantly higher amount of activity was demonstrated than with whole human blood. Both were significantly higher than the other materials tested here.

Additivity in stimulatory effect by the plasma fractions was tested by feeding the mixture of three fractions with erythrocytes. Enzymatic response to this mixture was greater than to any one fraction, but was
### TABLE I

Data on Protease Activity of Adult *Aedes aegypti* Females After Feeding on Various Food Materials.

<table>
<thead>
<tr>
<th>No. of Midguts*</th>
<th>Food Material</th>
<th>Protease Activity (Opt. Dens.)</th>
<th>Activity per Midgut</th>
<th>Average Activity per Midgut</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Starved</td>
<td>0.012</td>
<td>0.009</td>
<td>0.011</td>
</tr>
<tr>
<td>2.2</td>
<td>”</td>
<td>0.030</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Cells / saline</td>
<td>0.064</td>
<td>0.058</td>
<td>0.048</td>
</tr>
<tr>
<td>2.2</td>
<td>”</td>
<td>0.064</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Cells / serum dialyzate</td>
<td>0.065</td>
<td>0.059</td>
<td>0.072</td>
</tr>
<tr>
<td>2.2</td>
<td>”</td>
<td>0.135</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Whole sheep blood</td>
<td>0.227</td>
<td>0.206</td>
<td>0.209</td>
</tr>
<tr>
<td>2.2</td>
<td>”</td>
<td>0.065</td>
<td>0.211</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Whole human blood</td>
<td>0.532</td>
<td>0.242</td>
<td>0.242</td>
</tr>
</tbody>
</table>

* - Number of midguts in the final solution.
Relative Amounts of Protease Activity in Adult *Aedes aegypti* Females in Response to Various Food Materials Six Hours After Feeding.
not greater than the summed effect of the fractions (in excess of the value for erythrocytes alone). This is shown in Figure 2.

Relation of Activity to Amount of Blood Ingested

Table III presents the data of the quantitative effect of human blood on protease activity. The weight of blood per midgut is calculated from the weight of ingested blood divided by the number of mosquitoes fed that amount and not by the number of midguts in the final solution. These data were analyzed by the bivariate correlation coefficient test. The calculated correlation coefficient of 0.999 indicates a strong positive interaction between the weight of blood ingested by a mosquito and its subsequent protease activity. This rectilinear relationship is shown in Figure 3. This interaction remains whether a small number of mosquitoes was fed a large volume of blood or a large number of mosquitoes was fed small amount of blood.

A set of three tests was made for the presence of a possible zymogen-kinase relationship in these digestive enzymes. Nine mosquitoes were fed normally on the forearm and allowed to digest the meal for eighteen hours. The amount of blood was mixed with nine freshly homogenized midguts and incubated eighteen hours at 40°C, while the same amount of blood was incubated and then mixed with nine freshly homogenized midguts. The modified small sample technique for enzyme analysis was run on these three tests. There was no significant difference between the boiled blanks and runs in any test except normally fed individuals, indicating no zymogen-kinase reaction in vitro.
### TABLE II

Data on Protease Activity of Adult *Aedes aegypti* Females 18 Hours After Feeding on Various Food Materials

<table>
<thead>
<tr>
<th>No. of Midguts</th>
<th>Food Material</th>
<th>Protease Activity (Opt. Dens.)</th>
<th>Activity per Midgut</th>
<th>Average Activity per Midgut</th>
</tr>
</thead>
<tbody>
<tr>
<td>.77</td>
<td>Starved</td>
<td>0.008</td>
<td>0.010</td>
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</tr>
<tr>
<td>.77</td>
<td>&quot;</td>
<td>0.045</td>
<td>0.159</td>
<td></td>
</tr>
<tr>
<td>.77</td>
<td>&quot;</td>
<td>0.056</td>
<td>0.173</td>
<td></td>
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<td>.77</td>
<td>&quot;</td>
<td>0.065</td>
<td>0.214</td>
<td>0.024</td>
</tr>
<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.009</td>
<td>0.045</td>
<td></td>
</tr>
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<td>.19</td>
<td>&quot;</td>
<td>0.010</td>
<td>0.052</td>
<td></td>
</tr>
<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.004</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>.77</td>
<td>Cells / saline</td>
<td>0.133</td>
<td>0.172</td>
<td></td>
</tr>
<tr>
<td>.77</td>
<td>&quot;</td>
<td>0.072</td>
<td>0.235</td>
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<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.045</td>
<td>0.076</td>
<td>0.144</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.017</td>
<td>0.091</td>
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</tr>
<tr>
<td>.77</td>
<td>Cells / gum acacia</td>
<td>0.093</td>
<td>0.120</td>
<td>0.120</td>
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<tr>
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<td>Cells / serum dialyzate</td>
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<td>&quot;</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.018</td>
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</tr>
<tr>
<td>.77</td>
<td>Cells / serum proteins</td>
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<td>0.357</td>
<td>0.598</td>
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<tr>
<td>.77</td>
<td>&quot;</td>
<td>0.645</td>
<td>0.838</td>
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</tr>
<tr>
<td>.77</td>
<td>Cells / 0.6% fibrinogen</td>
<td>0.059</td>
<td>0.304</td>
<td></td>
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<td>.77</td>
<td>&quot;</td>
<td>0.056</td>
<td>0.291</td>
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</tr>
<tr>
<td>.58</td>
<td>&quot;</td>
<td>0.161</td>
<td>0.208</td>
<td>0.217</td>
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<td>&quot;</td>
<td>0.110</td>
<td>0.143</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.081</td>
<td>0.141</td>
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TABLE II (cont'd)

<table>
<thead>
<tr>
<th>No. of Midguts</th>
<th>Food Material</th>
<th>Protease Activity (O.D. Dens.)</th>
<th>Activity per Midgut</th>
<th>Average Activity per Midgut</th>
</tr>
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<tbody>
<tr>
<td>.77</td>
<td>Cells + 0.6% albumin</td>
<td>0.226</td>
<td>0.319</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.061</td>
<td>0.190</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.043</td>
<td>0.224</td>
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<td>&quot;</td>
<td>0.029</td>
<td>0.152</td>
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<td>&quot;</td>
<td>0.040</td>
<td>0.208</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.058</td>
<td>0.301</td>
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<td>&quot;</td>
<td>0.037</td>
<td>0.190</td>
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<td>.19</td>
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<td>0.037</td>
<td>0.145</td>
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<td>.19</td>
<td>&quot;</td>
<td>0.064</td>
<td>0.332</td>
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<td>.77</td>
<td>Cells + 0.6% gamma-globulin</td>
<td>0.413</td>
<td>0.536</td>
<td></td>
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<td>&quot;</td>
<td>0.449</td>
<td>0.583</td>
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<td>&quot;</td>
<td>0.062</td>
<td>0.322</td>
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</tr>
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<td>.19</td>
<td>&quot;</td>
<td>0.048</td>
<td>0.145</td>
<td></td>
</tr>
<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.039</td>
<td>0.200</td>
<td></td>
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<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.047</td>
<td>0.262</td>
<td></td>
</tr>
<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.051</td>
<td>0.266</td>
<td></td>
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<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.079</td>
<td>0.140</td>
<td></td>
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<tr>
<td>.19</td>
<td>Cells + albumin + gamma-globulin + fibrinogen</td>
<td>0.073</td>
<td>0.377</td>
<td>0.377</td>
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<tr>
<td>.77</td>
<td>Whole human blood</td>
<td>0.369</td>
<td>0.479</td>
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</tr>
<tr>
<td>.77</td>
<td>&quot;</td>
<td>0.530</td>
<td>0.688</td>
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<td>.77</td>
<td>&quot;</td>
<td>0.600</td>
<td>0.779</td>
<td>0.503</td>
</tr>
<tr>
<td>.19</td>
<td>&quot;</td>
<td>0.099</td>
<td>0.360</td>
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</table>

* - Number of midguts in the final solution.
Relative Amounts of Protease Activity in Adult Aedes aegypti Females in Response to Various Food Materials Eighteen Hours After Feeding.
TABLE III

Data of the Effect of the Weight of Whole Blood on the Protease Activity in Adult *Aedes aegypti*.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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<td>1.10</td>
<td>0.044</td>
<td>0.77</td>
<td>0.057</td>
<td>0.28</td>
<td>4.9</td>
</tr>
<tr>
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<td>0.050</td>
<td>0.77</td>
<td>0.065</td>
<td>0.55</td>
<td>8.5</td>
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<td>2.85</td>
<td>0.142</td>
<td>0.77</td>
<td>0.192</td>
<td>0.71</td>
<td>3.7</td>
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<tr>
<td>3.43</td>
<td>0.205</td>
<td>0.77</td>
<td>0.267</td>
<td>0.86</td>
<td>3.2</td>
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<td>4.75</td>
<td>0.272</td>
<td>0.77</td>
<td>0.353</td>
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<td>6.93</td>
<td>0.385</td>
<td>0.96</td>
<td>0.401</td>
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<td>7.01</td>
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<td>1.73</td>
<td>0.262</td>
<td>0.77</td>
<td>2.9</td>
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<td>7.25</td>
<td>0.417</td>
<td>0.77</td>
<td>0.542</td>
<td>1.81</td>
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<td>8.01</td>
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<td>0.77</td>
<td>0.505</td>
<td>2.01</td>
<td>4.0</td>
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<tr>
<td>8.30</td>
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<td>9.64</td>
<td>0.490</td>
<td>0.77</td>
<td>0.636</td>
<td>2.41</td>
<td>3.8</td>
</tr>
<tr>
<td>10.25</td>
<td>0.417</td>
<td>0.58</td>
<td>0.718</td>
<td>3.65</td>
<td>5.1</td>
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<tr>
<td>11.03</td>
<td>0.466</td>
<td>0.77</td>
<td>0.605</td>
<td>2.76</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* - Number of midguts in the final solution.
FIGURE 3

The Ratio of the Amount of Blood Ingested to the Protease Activity in Adult Aedes aegypti Females.
Results of Injected Series

Unfed mosquitoes injected with a mixture of saline and insect blood from blood-fed mosquitoes exhibited no increase in enzyme activity eight or ten hours after injection (0.014 optical density of the final solution).

Enzyme analyses were not possible on unfed mosquitoes injected with a mixture of saline and midgut contents of blood-fed mosquitoes, since three to four hours after injection many of the internal viscera including the midgut were digested, leaving the trachae seemingly intact.

Factors of Dilution

Assuming the average volume and the average weight of midgut tissue per female is 0.03 cubic millimeters and 0.03 milligrams, respectively (Fisk, 1950), the milligrams of female midgut tissue per milliliter of final solution read in the colorimeter can be calculated. Twenty midguts were homogenized and diluted to 3.5 ml. of saline and phosphate buffer, having the ratio of 5.71 midguts per milliliter brei. One half milliliter of the brei containing 2.86 midguts was diluted further to 3.25 ml. with trichloroacetic acid. Two and one-half ml. of the latter solution containing 2.2 midguts was diluted with an equal volume of sodium hydroxide. This resulted in a ratio of 0.44 midgut per milliliter of final solution. Multiplying the average value of the midgut tissue weight (0.03 mg.) by the fraction of the midgut in solution gives 0.0132 mg. female midgut tissue per milliliter of final solution in the colorimeter.

There are 25 mg. of azoprotein in each milliliter of stock solution. Since 0.25 ml. of substrate was used in each run, there
### TABLE IV

**Dilution Factors of Volume and Midgut Tissue**

<table>
<thead>
<tr>
<th>No. of Midguts Dissected</th>
<th>Final Volume Dilution</th>
<th>No. of Midguts in Final Solution</th>
</tr>
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<tr>
<td>20</td>
<td>1: 20,831</td>
<td>2.20</td>
</tr>
<tr>
<td>10</td>
<td>1: 41,665</td>
<td>1.10</td>
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<tr>
<td>9</td>
<td>1: 35,183</td>
<td>1.73</td>
</tr>
<tr>
<td>7</td>
<td>1: 45,236</td>
<td>1.35</td>
</tr>
<tr>
<td>5</td>
<td>1: 63,331</td>
<td>0.96</td>
</tr>
<tr>
<td>4</td>
<td>1: 79,165</td>
<td>0.77</td>
</tr>
<tr>
<td>3</td>
<td>1: 105,553</td>
<td>0.58</td>
</tr>
<tr>
<td>1</td>
<td>1: 316,665</td>
<td>0.19</td>
</tr>
</tbody>
</table>
(25)

would be 6.25 mg. per ml. of trichloroacetic acid filtrate. Two and
one-half milliliters of this filtrate plus 2.5 ml. of sodium
hydroxide would reduce the weight to 0.82 mg. of azocasein per
milliliter of final solution. By dividing the milligrams of
azoprotein per milliliter of solution by the milligrams of midgut
tissue per milliliter ($0.82 + 0.0132$) there would be 62.12 mg.
azoprotein per milligram of female midgut tissue per milliliter of
final solution. The dilution factors for other numbers of midguts
dissected and for the modified technique necessary for the computations
of enzyme activity units are given in Table IV.

Basis for Calculation of Activity Units

The readings from the Klett-Summerson photoelectric colorimeter
are easily changed to optical density by the multiplication by 0.002.
As previously stated, since the concentration relationships of
azocasein and its digestion products obey Beer's Law, optical
density can be substituted for protein concentration. The protease
activity may be expressed in terms of the velocity constant, $k$, of
enzyme reactions:

$$k = \frac{1}{t} \times 2.3 \log \frac{C_1}{C_2}$$

where $C_1$ and $C_2$ are the initial and final concentrations of protein
substrate respectively after $t$ minutes of digestion.

Since it is shown that the amount of enzyme present is universally
proportional to the time required to reach the desired end point, the
enzyme present may be measured by the "time value" (velocity constant)
Sumner and Somers, 1947). The velocity constant of an enzyme reaction
is then a function of enzyme concentration. The relationship between enzyme activity and enzyme concentration is rectilinear. In order to compute the velocity constant, the protein substrate concentrations must be calculated.

In the actual test, 0.25 ml. substrate was represented in each 0.75 ml. of incubated brei; therefore the ratio of the substrate to the solution is 1:3. Since an undigested, unprecipitated sample and a fully digested sample would give the same color intensity in the final solution to be read in the colorimeter, a blank was run on the dilution of azicasein substrate. This test gave an optical density of 7.36, the $C_1$ value or the original protein concentration. An optical density of 7.36 would correspond to complete digestion of the substrate.

The final protein concentration of the substrate, $C_2$, is found by subtracting the optical density of the trichloroacetic acid filtrate of each run from the $C_1$ value. This is permissible for the above reasons. The velocity constant can be computed easily by multiplying the difference of log. $C_1$ minus log. $C_2$ by 2.3 times 1/300 (1/t). An example can be drawn from the average value in optical density in Table II for the mixture of erythrocytes and non-dialyzable serum proteins (0.460). $C_1$ (7.36) minus this average value produces $C_2$ (6.90). Log. $C_1$ (0.8669) minus log. $C_2$ (0.8389) equals 0.023 which is multiplied by 2.3 times 1/300. The product is 0.000214, the velocity constant for the final solution.

Enzyme activity may be expressed in terms of the velocity
constant only if the rate of digestion of the substrate fulfills the requirements for a monomolecular reaction, i.e. if \( \log \frac{C_1}{C_2} \) is directly proportional to time. This fact was shown to be true of trypsin acting on azocasein by Charney and Tomarelli (1947). Enzyme activity for the original midgut solution may be obtained by multiplying the velocity constant by the total dilution factor of each enzyme solution. Using the velocity constant derived above and the total dilution factor for the four midguts as given in Table IV (1: 79, 165), the velocity constant of the original midgut solution in this example is 16.94.

The activity units (actually velocity constants) are arbitrary and are not to be confused with the Willstater units for trypsin activity. In spite of the calculations required, these units have no more merit than the optical densities used to express protein concentration and trypsinase activity. Therefore they have not been calculated at this time except for the above example.
DISCUSSION

In the past few years much research has been devoted to various enzymes. Enzymes have been implicated in physiological processes from birth control to the mode of action of some new organic insecticides. Recently it has been proven with direct evidence that an enzyme-substrate complex which was proposed from studies of the kinetics of enzyme-catalyzed reactions actually exists. Through research with inhibited enzyme systems it is possible to conclude that the substrate and enzyme are very specific in nature and locality. As a result of the complex formed between the enzyme and the substrate the substrate becomes more active chemically.

The vast majority of enzyme-catalyzed reactions require other substances than the enzyme and substrate before chemical changes can occur. There are "activators", which are accessory substances required before the enzyme can activate its substrate, and "coenzymes or prosthetic groups" which play some part in the reaction catalyzed by the enzymes. Coenzymes are loosely and temporarily attached to the substrate whereas the prosthetic groups are relatively firmly fixed to the protein partners, usually among enzymes concerned with oxidation and reduction processes (Baldwin, 1952).

Insect proteases consist of proteinases, which act on natural or high molecular weight proteins, and a group of peptidases by which the products of protein digestion are further hydrolyzed. The proteinase is always of the trypsin-type since the optimum
pH is too high for pepsin (Scheer, 1914). Protease liberates peptones, polypeptides, dipeptides, and amino acids from the native proteins. The first three serve as substrates for the various peptidases: carboxyolypeptidase, aminopolypeptidase, and dipeptidase (Wigglesworth, 1950; Roeder, 1953). Wigglesworth (1950) suggests that proteinases are extracellular and peptidases are endocellular so that final hydrolysis of proteins may take place within the midgut epithelium. Scheer (1914) and Baldwin (1952) indicate that all true insect blood digestion is extracellular and infer that endoenzymes are more closely associated with metabolic processes, such as synthesis, autolysis, and growth.

Although insect proteases are more similar to vertebrate trypsin than other proteases, several differences are apparent. Insect protease will attack native protein, but of trypsin Sumner and Somers (1917) write "...unlike pepsin, it (trypsin) is not an enzyme of first attack and, in several instances, it possesses only feeble action on native proteins... These proteins are readily digested by trypsin once they have been denatured by heat or other means." Close comparisons between insect and vertebrate enzymes are difficult since there are no highly purified crystalline preparations of insect digestive enzymes (Roeder, 1953). Many enzymes present in insects are similar to those in mammals and other vertebrate forms. Perhaps the differences between these enzymes can be resolved by a more thorough research program on enzyme specificity and activators.

The mere presence of an enzyme in the digestive tract of an insect does not irrevocably establish its substrate as a food of
the particular insect (Fraenkel, 1910), although in insects the correlation between food and enzymes to digest them is good. MacGregor (1931) reported that male mosquitoes, force-fed by the "capillary-tube" technique of unsheathing the fascicle, were capable of digesting blood within the same period of time required by female mosquitoes. This technique was tried, but the author was unsuccessful in his attempt to induce either sex of mosquitoes to ingest blood in this manner.

Schlottke (1937 a,b,c) was the first to study the effect of various diets on the insect digestive enzyme activity. He was able to demonstrate quantitative changes in enzyme concentrations after feeding on different diets. There were reported changes in enzyme activity at various times after feeding. Enzyme activity was found to be lower in starved insects than in fed insects, but there was a residual value for individuals that were starved (Day and Powning, 1949; Fisk and Shambaugh, 1952).

In *Aedes aegypti* there is a pronounced initial drop in rate of proteolytic enzyme activity immediately following a blood meal to five minutes afterwards. The rapid rate of increase after five minutes is as great as the initial drop. The rate of activity gradually falls off, but the difference in amount of activity increases with time until 18 hours after feeding, at which time both rate and amount of activity decline. After a meal of sugar solution there is an immediate increase and preponderance of protease activity over that resulting from a blood meal for a little over an hour after feeding, but the rate of increase in activity is smaller.
These values quickly resume a residual position (Fisk and Shambaugh, 1952). From his studies on proteolytic digestion in *Aedes aegypti*, Fisk (1950) concluded that the optimum pH for midgut protease was 7.8, using serum albumin as a substrate. He also suggests a coagulation or agglutination of blood which presumably denatures the proteins prior to the activity of the proteases.

Shlenova (1933) considers the optimum temperature for the blood digestion in *Anopheles maculipennis* to be 20° C. The rate of digestion is increased up to temperatures of 30° C., but then it is retarded. High humidity accelerates digestion at temperatures above 15° C. with the optimum humidity range 90% to 100%. By use of the precipitin test for blood, West and Eligh (1952) were able to conclude that light intensity may increase the rate of digestion by increasing the activity of the whole mosquito. After eight days at 11° C. or one or two days at 27° C., it was possible to get a precipitin test for undigested blood. This test is of no value for digestion products of blood. Fisk and Shambaugh (1952) estimated that complete digestion of blood in a living mosquito requires about 48 hours after feeding when held at 27° C.

Although in vertebrates, food and its products may act as enzyme stimulators or the stimulus may be mediated by hormones or by nerves, only the former two processes have been reported in insects. Day and Powning (1949) were able to demonstrate histologically on *Tenebrio molitor* the effect of an injected hormone (secured from fed insects) on regenerative cells (nidi) of the midgut. The actual gland secreting the "midgut regeneration stimulation factor" is not
known. In *Modnius prolilus* Wibblesh worth (1915) states, "... Associated with the development of eggs in the adult female there is a much more rapid digestion of the intestinal contents. Whether this is a direct effect of the hormone on digestion and metabolism or whether it is an indirect effect consequent upon the demands of the developing ovaries has not been determined."

From a series of experiments in which a mixture of mosquito saline and blood from insects previously fed a blood meal was injected into unfed mosquitoes, Shamburgh (1951) concluded that in *Aedes aegypti* protease elaboration was stimulated not by hormones but by secretagogues from the blood or its products. Further tests along the same line, as described in the previous section, support this theory since there was no essential increase in enzyme activity above the residual value eight or ten hour after injection. To test for conceivable precursors or zymogens present in the midgut upon which an activator or kinase feasibly present in the blood could act, unfed individuals were tested against blood-fed individuals, as described in the previous section. The results indicate that no zymogen-kinase relationship with blood exists. Fisk (1950) reported that no combination of crops, salivary glands, and empty midguts showed any measurable increase in protease activity. However, his tests were run after two hours incubation.

Since there was no evidence for activation by endocrine inducement of protease activity nor for a zymogen-kinase relationship, one can consider the effect of the various fractions of blood on protease activity as given in Tables I and II. The residual
value is given for unfed or blood-starved individuals. The effect of cellular proteins in an inert medium (erythrocytes in saline) is somewhat greater than the residual value. Both the dialyzable and non-dialyzable portions of blood were tested. The dialyzable portion mixed with erythrocytes was one and one-half times as effective in enzyme stimulation as the mixture of erythrocytes in saline. This would indicate a dialyzable weak stimulatory factor in the plasma of blood. The non-dialyzable plasma proteins and erythrocytes resuspended in saline intensified the protease activity to a level four times that found for erythrocytes in saline, indicating a strong stimulatory effect. There was no principle difference between the digestive activities of those mosquitoes fed whole human blood and those fed whole sheep blood.

Inasmuch as the non-dialyzable fraction of the plasma (mostly protein) stimulated the most activity, various fractions of plasma proteins were fed separately and once collectively. Fibrinogen excited an elaboration of enzymes comparable to that of the plasma dialyzate. Plasma albumin increased the enzyme activity more than fibrinogen but not as much as gamma-globulin. The mixture of the three 0.6% solutions of proteins and erythrocytes stimulated more activity than any other one fraction. However, this amount of activity was less than that of the sum of the differences of each protein fraction minus the value for the cellular proteins. This discrepancy is probably due to an error in sampling (only one test was possible) or it is derivable from the effect of having the concentration in the composite solution higher than in the
There exists a strong interaction between the amount of blood ingested by female mosquitoes and subsequent protease activity. The calculated correlation coefficient was 0.244. This would lead one to the inference that the blood ingested serves to increase the amount of protein substrate for the midgut proteases. This relationship is reminiscent of the influence of substrate concentration on enzyme activity when the reaction rate is monomolecular.

Although females of some species of mosquitoes are able to lay autogenous eggs, Aedes aegypti require a blood meal first. Roy (1936) postulates that for the first batch of eggs 0.82 mg. of blood, and 0.45 mg. for the second batch of eggs, is needed for follicle stimulation and an excess is used in egg production. Assuming that such factors exist, from the behavior of the ovaries he concluded that the "...same element exercises dual function..." Woke (1937 a) studied the comparative effects of various vertebrate bloods on egg production of Aedes aegypti. Blood from the turtle, frog, canary, rabbit, and guinea pig were better for egg production than blood of man or donkey. Woke (1937 b) succeeded in securing viable eggs from mosquitoes fed whole, defibrinated, decalcified, or heparinized blood; erythrocytes, plasma, serum, and hemoglobin. Greeberk (1951) received 16 times the number of eggs from mosquitoes fed defibrinated sheep blood than from mosquitoes fed washed sheep erythrocytes. Inorganic salts, a series of vitamins, and glucose failed to increase oviposition. Proteins increased oviposition.
Proteins could be adequately replace by dl-isoleucine but nine other amino acids failed.

In the past two decades much valuable information has been accumulated for the understanding of the physiology and infectivity of malarial parasites through the use of bird malarial parasites and their vectors. MacGregor (1931) reported that avian malarial parasites failed to develop if ingested into the diverticula. This fact would suggest that some reaction in the midgut, perhaps some process of digestion, was necessary for development. Nicolaew and Yakowlawa (1929) reported that the parasite macrogamete was fertilized in both infection-resistant and infection-susceptible strains of mosquitoes. Studying such strains of mosquitoes Huff (1927, 1934) concluded that immunity was not explained by differences in digestion discernible by histological methods. He noted also that not all of the ookinetes succeed in penetrating the midgut wall. The responsible mechanism is not known. Other than genetic variation the degree of infectivity and oocyst development are greatly influenced by temperature but not by humidity nor by activity nor age of the mosquito (Huff, 1941 a,b).

The number of oocysts developed in the mosquito midgut is not directly correlated to the number of infective cells in the meal (Hovanitz, 1947). This would indicate factors limiting infectivity other than exposure. The greater the number of oocysts, the greater the number of eggs laid, but an increase in egg production does not necessarily increase oocysts formation. This was interpreted to be correlated with the absorption of blood after digestion. In a series
of studies on the avian parasite *Plasmodium gallinaceum*, Eyles (1951, 1952 a,b,c) published comparisons of susceptibilities of various mosquito species, characteristics of mosquito infection, and factors in the blood of the vertebrate host associated with mosquito infectivity. He was able to demonstrate that serum alone was sufficient for full development of the parasite, but one-quarter development could occur on erythrocytes suspended in saline. The dialyzable portion of the plasma has no effect. Bovine plasma albumin promted mosquito infections in concentrations less than six percent when compared with physiological saline alone, but infections were less intense than those resulting from deforininated blood.

There are still undetermined factors in the vertebrate blood affecting infection. Transfusion of normal blood into highly parasitized chickens was found to enhance infectiousness of the blood in two or three attempts. Size of oocysts, as well as their number, was found to be effected by the intensity of infection in the chicken (Cantrell and Jordan, 1946; Eyles, 1952 b).

Studies on the physiological pathology of malaria in the intermediate host show oncreases in the blood of cellular proteins (after cellular disintegration), fibrinogen, and euglobulin, with an accompanying decrease in plasma albumin. In view of those facts and the data presented in the above discussion separately linking malarial infection and protease elaboration with plasma proteins, it is possible to suggest a stronger interaction between processes affecting digestion and infectivity than earlier supposed.
SUMMARY

Results of previous studies which concluded that the stimulation of proteolytic enzymes in the midgut of *Aedes aegypti* is secretogogue in nature served as a basis for this research. The technique of feeding adult female mosquitoes blood substitute solutions through warmed membranes is described.

A series of mosquito injection tests supports the previously proposed theory of protease stimulation by secretagogues. Tests for conceivable zymogens present in the midgut stimulated by a kinase carried in the blood (similar to the trypsinogen-enterokinase relationship of vertebrates) were negative. Various blood fractions were tested for possible secretagogue influences. In order for blood substitute solutions to be dispatched primarily to the midgut where protease elaboration is confined, it was expedient to mix them with thrice-washed sheep erythrocytes.

A residual value of protease activity is given for unfed, or blood-starved, individuals. The effect of cellular proteins in a relatively inert medium (sheep erythrocytes in physiological saline) is somewhat greater than the residual value. Both the dialyzable and non-dialyzable portions of blood were tested. The dialyzable portion mixed with erythrocytes was one and one-half times as effective in enzyme stimulation as the mixture of erythrocytes in saline. This would indicate a weak dialyzable stimulatory factor in blood plasma. However, the non-dialyzable plasma proteins and erythrocytes resuspended in saline intensified enzyme activity to a height four times that of erythrocytes in saline. Evidently the more important stimulatory
factors are in the non-dialyzable plasma fractions.

The effect of three plasma protein fractions at a concentration of 0.6% was tested. Fraction I (fibrinogen), normally 6% of plasma proteins, stimulated a secretion of enzymes comparable to that of the plasma dialyzate. Fraction V (albumin), normally 48% of plasma proteins, provided an increase in activity above that of fibrinogen. Fraction II (gamma-globulin), normally 6% of plasma proteins, stimulated a secretion greater than the sum of the fractions I and V. Enzymatic response to the mixture of these three fractions of plasma proteins was greater than to any one fraction, but was not greater than the summed effect of the separate fractions (in excess of the value for erythrocytes alone).

Through a series of experiments utilizing individual weights, it was possible to discover a positive correlation between the amount of blood ingested by female mosquitoes and the subsequent protease activity of their midguts. This would lead to the conclusion that an increase in ingested blood increases the amount of the protein substrate for the midgut proteases.

Discussions of the effect of the blood fractions fed to mosquitoes upon the degree of oviposition or infectivity with malarial parasites are given. From this discussion intimately linking malarial infection and protease elaboration separately with plasma proteins, it is possible to suggest a stronger interaction between processes affecting digestion and infectivity than supposed earlier.
LITERATURE CITED


I, George Franklin Shabaugh, was born in Columbus, Ohio November 3, 1928. I received my primary and secondary school training in the public schools of Columbus, Ohio. My undergraduate college training was obtained at Wilmington College, Wilmington, Ohio, from which I graduated with the degree Bachelor of Arts in 1950. I entered The Ohio State University the same summer and received the degree Master of Science in 1951. During 1950-51 I was appointed Research Fellow in the Department of Zoology and Entomology under Dr. Frank H. Fisk. From 1952 until the completion of the requirements for the degree Doctor of Philosophy I was appointed Research Fellow in the same department under Dr. Ralph H. Davidson and Dr. Frank W. Fisk.

I am a member of the Society of the Sigma Xi, Chi Beta Phi, Entomological Society of America, Ohio Academy of Science, and the American Mosquito Control Association.