

PROTEASE ACTIVITY IN ADULT *Aedes aegypti* MOSQUITOES AS RELATED TO FEEDING¹

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The importance of mosquitoes as disease vectors is now well established, but much remains to be learned about the fundamental processes of digestion in mosquitoes, especially as they relate to the transmission of these diseases. The studies reported here deal with the stimulation of proteolytic enzyme activity in the adult female yellow-fever mosquito, *Aedes aegypti* L., in relation to the interval of time after feeding and other factors.

METHODS

The *Aedes aegypti* mosquitoes used in these tests were obtained as needed from the colony maintained by an Ohio State University Research Foundation Project sponsored by the U. S. Public Health Service. Female mosquitoes four to six days after emergence were used, since at that age they feed most readily on blood and have, by that time, disposed of any larval food which remained in the midgut at emergence (Fisk, 1950). The insects were transferred to a lamp chimney covered at the top with gauze and resting on a cross-slitted dental rubber dam held in place by an embroidery hoop.

Blood Feeding Techniques

Blood feeding was induced by placing the bare forearm on the gauze over the lamp chimney. The female mosquitoes were attracted to the arm and fed through the gauze. As each one became partially engorged with blood it was withdrawn from below with an aspirator inserted through the cross-slits and placed in a separate three-inch shell vial which was then stoppered with a wad of cotton. The time of feeding for each specimen was noted. After a series of vials had been filled they were transferred to the insectary held at about 80° F and 50 percent relative humidity.

The midguts were dissected from the blood-fed females after a given interval of time had been allowed for normal digestive processes. These time intervals varied from zero to 48 hours as indicated in table 1. Prior to dissection a mosquito was momentarily stunned by a sharp rap of the vial against the palm of the hand. It was then placed on a paraffin covered single concavity slide and decapitated. The midgut was teased from the abdomen and separated from other viscera in a few drops of Levy's mosquito saline. The midguts were accumulated in lots of 20 in a small shell vial resting on ice and containing a drop of chilled saline. The vial was then labelled and placed in a deep freezer at -15° C until needed.

Sugar Solution Feeding Technique

For the sugar-fed series the usual sugar solution and water was withheld from the stock cage the night before. This was necessary to obtain a quick feeding response and to be sure of the proper timing of the feeding. A pledget of cotton saturated with prewarmed 5 percent sucrose solution was placed on the gauze of the lamp chimney. Replete females were removed and placed in individual vials just as with the blood-fed series. Dissections were made just as described for the blood-fed mosquitoes.

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Injection Technique

A series of mosquitoes was blood-fed and placed in the insectary for from five to twelve hours. Each female was then anesthetized with carbon dioxide gas and its head crushed. With the body on a block of paraffin the abdomen was then carefully teased apart in the region of the fourth or fifth segment. Care was exercised in order not to puncture or break the digestive tract in any way. About 2.5 microliters of Levy's saline was dropped onto the exposed midgut from a $\frac{1}{4}$ cc tuberculin syringe. The resulting mixture of haemolymph and saline was then picked up by a mouth syringe and injected into a previously anesthetized unfed female *Aedes*. The mouth syringe consisted of a plastic mouthpiece, a small bore rubber tube six inches long, and a three inch long glass tube ending in a fine capillary "needle" tip. The mosquito to be injected was held securely in a specially constructed holder. The injection tip was inserted into the thoracic region at the suture between the meso- and metapleuron and forced posteriorly into the abdomen. As the mixture entered the body the abdomen became noticeably distended. Immediately after injection the mosquito seemed to be in a state comparable to shock, from which it later recovered. Each injected mosquito was placed in a separate shell vial and returned to the insectary for one, two, or five hours after which it was dissected and the midgut removed as previously described. Any mosquitoes which died or which showed injury to the alimentary canal at the time of dissection were discarded. As a check on the technique several mosquitoes were anesthetized, injected with saline, and hours later, dissected.

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 activity of the enzyme source and its measurement (as optical density) serves as the basis of this technique.

The substrate, sulfanilimide-azocasein, was first prepared as described by Charney and Tomarelli (1947). A stock solution was then made up to contain 2.5 g azocasein and 0.5 g of sodium bicarbonate per 100 ml. The pH was adjusted to 8.3 and the solution stored at 3° C. The procedure for each enzyme run began with the removal of a vial of 20 dissected-out midguts from the deep freezer. These were homogenized without an abrasive. 1.5 ml of Clark and Lubs standard phosphate buffer, pH 7.8 (Morrow and Sandstorm, 1935), and enough Levy's saline to bring the total volume to 3.5 ml were added to the resulting brei. pH 7.8 has been shown by Fisk (1950) to be optimum for *Aedes aegypti* protease. The mixture was stirred, then 3.0 ml were pipetted into six micro test tubes, 0.5 ml per tube. Two tubes were placed in a boiling water bath while the other four were put into the deep freezer. After 20 minutes boiling, the boiled blanks were placed in the deep freezer with the other tubes until they were approximately the same temperature, (10 minutes). The series then was removed from the freezer and 0.25 ml azocasein substrate added to each tube. The breis were stirred again and placed in a constant temperature bath at $39.6 \pm 0.5^\circ$ C for five hours incubation.

The incubated runs and blanks were removed from the water bath and emptied into six numbered centrifuge tubes, each containing 0.5 ml of 10 percent trichloroacetic acid. Each incubation tube was rinsed into the appropriate centrifuge tube with two more milliliters of trichloroacetic acid. The precipitated protein was separated by centrifuging ten minutes in a Sorvall angle centrifuge at 5500 rpm.

Next, 2.5 ml of supernatant from each tube was transferred to a calibrated Klett colorimeter tube, followed by the addition of 2.5 ml 0.5 N sodium hydroxide to each Klett tube. This shift in pH served to intensify and deepen the color. Optical densities were determined by means of a Klett-Summerson photoelectric colorimeter, using the 440 millimicron blue filter.

The enzyme assay technique was checked by the use of 0.1 percent, 0.01 percent, and 0.001 percent solutions of Coleman-Bell commercial trypsin.

RESULTS

The function of the first series of experiments was to study quantitatively protease activity in the mosquito at various times during digestion of a partial blood meal. The amount of protease activity determined was the amount of

TABLE 1

Data on protease activity of the blood-fed series of Aedes aegypti at various intervals of time after feeding.

Interval of time after feeding	Number of runs	Average optical density	Percent azoprotein hydrolysis	Activity units	Milligrams protein released	Percent efficiency
0-2 min.	4	0.0158	1.16	0.24	0.0253	8.43
5 min. (\pm 1 min.)	8	0.0068	0.50	0.15	0.0109	3.63
15 min. (\pm 2 min.)	4	0.0256	1.88	0.57	0.0410	13.33
30 min. (\pm 3 min.)	8	0.0264	1.94	0.59	0.0423	14.10
1 hr. (\pm 5 min.)	4	0.0296	2.18	0.65	0.0475	15.83
2 hrs. (\pm 10 min.)	8	0.1094	8.04	2.47	0.1753	58.43
4 hrs. (\pm 20 min.)	4	0.2170	16.00	5.09	0.3488	116.27
8 hrs. (\pm 20 min.)	8	0.4320	31.76	11.18	0.6924	230.80
12 hrs. (\pm 20 min.)	4	0.5146	37.84	13.91	0.8249	274.97
18 hrs. (\pm 20 min.)	8	0.6324	46.50	18.30	1.0137	337.90
24 hrs. (\pm 30 min.)	12	0.5996	44.09	17.01	0.9812	320.40
48 hrs. (\pm 30 min.)	8	0.3758	27.63	9.46	0.6023	200.77

TABLE 2

Data on protease activity of the sugar-fed series of Aedes aegypti at various intervals of time after feeding.

Interval of time after feeding	Number of runs	Average optical density	Percent azoprotein hydrolysis	Activity units	Milligrams protein released	Percent efficiency
Unfed	11	0.0274	1.99	0.59	0.0434	14.47
5 min. (\pm 1 min.)	4	0.0296	2.16	0.65	0.0471	15.70
30 min. (\pm 3 min.)	4	0.0444	3.65	0.96	0.0796	26.53
4 hrs. (\pm 20 min.)	4	0.0244	1.79	0.52	0.0390	13.00
8 hrs. (\pm 20 min.)	4	0.0320	2.35	0.70	0.0512	17.07
18 hrs. (\pm 20 min.)	4	0.0310	2.28	0.68	0.0497	16.57
24 hrs. (\pm 20 min.)	4	0.0250	1.84	0.54	0.0401	13.37

available protease at the time of dissection, minus losses due to the technique. The amount or condition of the blood meal within the midgut at the time of dissection had no effect on the results since the blood protein would be removed by the trichloroacetic acid precipitation and any soluble colored materials would be equal in both blanks and runs. The results are given in table 1 and figure 1. They show a sharp drop in activity lasting but a few minutes, followed by a steady rise lasting several hours with the peak of activity at 18 hours after feeding. It will be noted that the lowest value obtained was five minutes after a blood meal. This low value is significantly lower than that obtained from unfed mosquitoes.

The amount of protease activity shown by the midguts of unfed mosquitoes shall be referred to as the residual value.

The data for the sugar-fed series (table 2, fig. 1) show an initial increase in activity during the first half an hour. The protease activity then returns to and remains within the range of the residual value. Figure 1 shows that the effect of sugar solution feeding on protease activity is transient and never approaches the stimulation afforded by the adequate substrate, blood. Neither does it cause the initial drop in activity noted with blood. The effect of sucrose feeding on invertase activity was not studied.

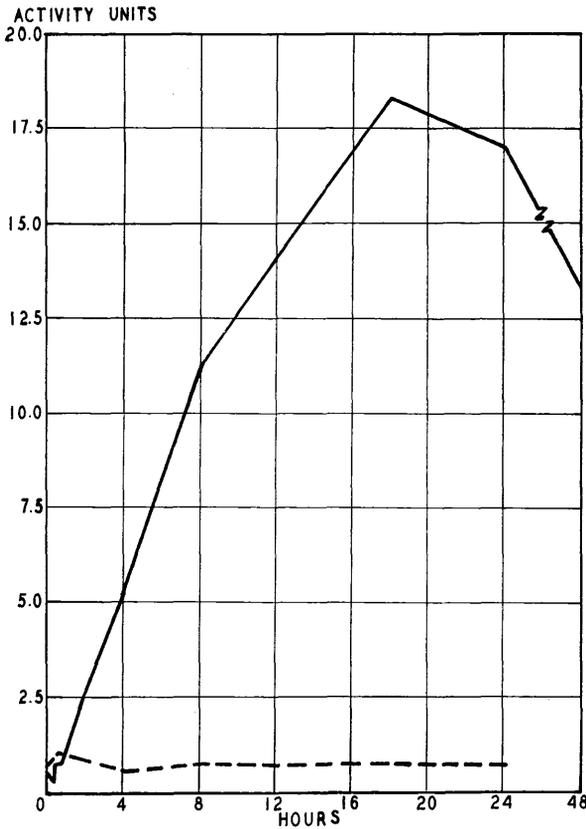


FIGURE 1. Differences in amount of activity of proteolytic enzymes of *Aedes aegypti* at various intervals after feeding on blood or sugar. Solid line: blood-fed mosquitoes; broken line: sugar-fed mosquitoes. Source: Tables 1 and 2.

Basis for Calculation of Activity Units

The readings from the Klett-Summerson photoelectric colorimeter are easily converted to optical densities by multiplying them by 0.002 (Hawk, Oser, and Summerson, 1947). These optical densities can be substituted for protein concentrations as shown by Charney and Tomarelli (1947). The protease activity may then be expressed in terms of the velocity constant, K, of enzyme reactions.

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

where C_1 is the initial protein substrate concentration and C_2 is the final protein substrate concentration after t minutes of digestion.

The amount of enzyme present may be measured by the "time value" (velocity constant) (Summer and Somers, 1947). The velocity constant is a function of enzyme concentration. In order to compute the velocity constant, the protein substrate concentration must be calculated.

Since an undigested, unprecipitated, 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 azocasein substrate. This test gave an optical density of 1.36 for the C_1 value or the original protein concentration. This value would correspond then to 100 percent substrate hydrolysis.

The final protein concentration, C_2 , is found by subtracting the optical density of the trichloroacetic acid filtrate of each run from the C_1 value. The velocity constant is easily computed after the substitution of the C_1 and C_2 values into the former equation.

Since enzyme activity may be expressed in terms of the velocity constant, enzyme activity of the original midgut contents may be obtained by multiplying the velocity constant by the total dilution factor of the enzyme dispersions, 1:8,749.5. The activity units are arbitrary and are not to be confused with Willstätter units. Percent of substrate hydrolysis is based on the ratio of the optical density of the final solution to the optical density of undigested azocasein (1.36). Activity units and percent hydrolysis are found in tables 1 and 2 and discussed in more detail by Shambaugh (1951).

Factors of Dilution

Assuming the average volume and the average weight of midgut tissue per female to be 0.03 cubic millimeters and 0.03 milligrams, respectively (Fisk, 1950), the milligrams of female midgut tissue per milliliter can be calculated. In our technique there was a ratio of 0.038 midgut per milliliter of final solution in the colorimeter. Multiplying the average value of midgut tissue weight (0.03 mg) by the fraction of midgut in solution gives 0.00114 mg female midgut tissue per milliliter of final solution.

Since there are 25 mg azoprotein in each milliliter of stock solution, there would be 0.82 mg of azoprotein per milliliter of final solution, taking dilution into consideration.

Injection Experiments

In this series of experiments a mixture of saline and haemolymph from blood-fed mosquitoes or saline alone was injected into unfed individuals which were later dissected for enzyme assay. The results as shown in table 3 were completely

TABLE 3

Data on protease activity of Aedes aegypti at intervals of time after the injection of haemolymph of blood-fed individuals into unfed individuals.

Number of runs	Time after feeding	Interval after injection	Average optical density	Percent azoprotein hydrolysis	Activity units
4	check	2 hrs.	0.0240	1.77	0.52
4	5 hrs.	2 hrs.	0.0180	1.32	0.39
4	12 hrs.	1 hrs.	0.0180	1.32	0.39
4	12 hrs.	5 hrs.	0.0156	1.15	0.33
4	15 hrs.	2 hrs.	0.0260	1.91	0.56

negative, that is the protease activity values obtained did not exceed the residual values. The significance of these results will be discussed later.

DISCUSSION

Before discussing the results of the present study it should be pointed out that the work of Fisk (1949, 1950) has provided the starting point for our research. He determined for *Aedes aegypti* the normal pH of the alimentary canal, the source and optimum of pH of the protease, and the fact that protease activity can be demonstrated one to two hours after a blood meal but not after feeding on sucrose solution or in unfed female mosquitoes. The work of Day and Powning (1949) has also provided a strong stimulus to our efforts. They employed modern techniques to study several aspects of digestion in *Blatella*, *Periplaneta*, and *Tenebrio*. Referring to their results they state: "Some of the data . . . are at variance with current concepts and others represent new aspects of the processes of digestion in insects." Their challenging paper points up sharply the lacunae in our knowledge of this field.

The first use of an azoprotein in insect protease studies was by Day and Powning (1949). This technique first tried with duodenal juice (Charney and Tomarelli, 1947) is rapid, accurate, and simple. It is well suited to insect protease studies. In a more recent variation Tomarelli, Charney, and Harding (1949) employ azoalbumin instead of azocasein so that the substrate may be used in both acid and alkaline solutions.

Enzyme Stimulation Following Feeding

Biedermann (1898) was probably the first to note increases in digestive enzymes following feeding. Other authors have noted cyclical changes in midgut epithelium, such as cytoplasmic granule formation or "hypersecretion," presumably due to intensified secretory activity. Day and Powning (1949), however, contend that these changes represent the destruction of the older cells and not secretion. They found the highest enzyme concentrations in the areas of uniform cytoplasm, the so-called "resting epithelium." Actual increases in enzyme activity were noted by Schlottke (1937a, b, c) in *Carabus auratus* (Coleoptera), *Tettigonia cantans* (Orthoptera), *Blatta* (= *Periplaneta*) and *Blatella*. The latter two species were also studied extensively by Day and Powning (1949). The insects just referred to, except the predacious Carabid, are omnivorous feeders in which some food is almost always present in the crop or midgut. All showed:

- (a) A decrease in enzyme activity following starvation.
- (b) An increase in all enzymes following a brief (10 minute) feeding. The stimulation was not immediate, but clearly evident in half an hour and continued for some hours, (roughly three to eight).
- (c) All enzymes were stimulated, irrespective of the food.
- (d) There was a marked decrease often below the starvation level, of a given enzyme following a longer (half hour) feeding of its substrate. Depletion of the enzyme by its substrate was cited as the chief cause of this phenomenon.

The results of the present paper and those of Fisk (1950) are based on an insect with entirely different feeding habits. The female mosquito may feed on soluble carbohydrates at frequent intervals but her primary food is blood which, though ingested in a minute or two, may be held for two days or more while it is being digested. For comparison, the results with *Aedes aegypti* may be summarized as follows:

- (a) The residual value of protease activity in a blood-starved (e.g. blood-unfed) mosquito is very low. (It was not detected by Fisk, 1950).
- (b) A brief feeding (the only type) on blood produced an immediate decrease in protease below the residual value followed by a great increase which was highest at about 18 hours and still evident at 48 hours.

- (c) The effect of blood-feeding on other enzymes was not studied, but the effect of sugar solution feeding on trypsin was that of an initial moderate increase noted in the first half hour but entirely dissipated within four hours. Succeeding measurements showed only residual activity.
- (d) Continued feeding ($\frac{1}{2}$ hour) was not possible with *Aedes*.

Several contrasts are apparent between the results with *Aedes* and with the omnivorous, frequent feeders. For instance, different foods produced different patterns and degrees of protease stimulation. In this connection it may be recalled that in mosquitoes blood and sugar solutions are ingested quite differently, blood going directly to the midgut, while sugar solutions and water go first to the ventral diverticulum or "crop." The high degree and long duration of protease stimulation following a blood meal is another contrast. This pattern cannot be true of all blood sucking insects, however, since preliminary tests in our laboratory with the bedbug, *Cimex lectularius*, showed only a weak trypsin-like activity for periods up to 12 hours after feeding.

The initial drop in protease activity immediately following the blood meal may correspond to the depletion of enzyme by its substrate noted by Day and Powning following continued feeding. In their tests the insects were slow to recover from these depletions, while with *Aedes* the "depletion" is made up within half an hour's time despite the continued presence of excess substrate. Schmitz (1938) has described an anti-trypsin in human blood which may temporarily neutralize the mosquito trypsin more rapidly than would be possible by a simple depletion effect.

Mechanism of Enzyme Stimulation Following Feeding

Three possible mechanisms of enzyme stimulation due to feeding have been suggested for insects: (a) secretagogue; the foodstuff itself or its products may chemically stimulate secretion; (b) nervous; the act of feeding, detection of food, or the presence of food may set up a nerve reflex to which the secretory cells will respond; (c) hormonal; like nervous mechanism except that feeding results in production of a hormone which reaches the digestive tract through the haemolymph.

In man all three mechanisms operate in combination but there is no evidence of this in the insects studied. Although Fisk (1950) suggested the possibility of nervous intermediation this seems unlikely in view of the delayed responses noted in every case. Also, Day and Powning have shown that the midgut of *Periplaneta* and the caecae of *Periplaneta* and *Blattella* lacked nerves.

On the basis of simplicity the secretagogue hypothesis would be favored over the hormonal theory, but no direct evidence to support it has yet been given. The injection experiments reported here indirectly favor the secretagogue theory inasmuch as they were designed to confirm the hormonal hypothesis but failed to do so.

The hormonal mechanism is favored by Day and Powning (1949). They point out that the gastric caecae in *Periplaneta* are poorly fitted for the diffusion of food from the gut, but yet they begin to secrete enzymes sooner than the midgut presumably because the hormone flowing back from the head region reaches them first. These authors made injection experiments with both *Periplaneta* and the beetle, *Tenebrio molitor*, in which blood from a fed individual was injected into a starved one and any increases in cell division of the nidi (young secretory cells) noted. Inconclusive data were secured from *Periplaneta* but stimulation was noted in *Tenebrio*. The stimulation was more rapid and more transient than that following feeding but this might be due to the relatively small amount of hormone transferred by the injection. As previously noted, our injection experiments with *Aedes* were all negative.

Quantitative Measurement of Enzyme Efficiency

In order to correlate the efficiency of enzyme solutions under varying conditions with earlier work, it will be necessary to interpret results and reduce them to common terms, to note similarities and differences in technique and enzyme concentration.

Because the enzyme:substrate ratio is quite important to insure enzyme stability and rate of activity up to a point, the weight of protein per midgut per incubation tube was found. Fisk (1949) describes a run having 1 mg protein per midgut per tube. In this study the ratio is 2.18 mg azoprotein/midgut/tube.

Percent efficiency may be calculated by dividing the maximum weight of azoprotein which could be digested by a hypothetical mosquito by the actual amount of azoprotein digested. Assuming that the average mosquito weighs 1.5 mg and ingests its weight in blood (Fisk, 1950) and that whole vertebrate blood contains 20 percent proteins, the maximum amount of protein ultimately ingested is 0.3 mg per midgut. By multiplying the percent hydrolysis of the substrate by the milligrams of azoprotein/midgut/tube (percent hydrolysis X 2.18), one obtains the actual amount of azoprotein in milligrams released per midgut in each test.

Fisk (1949) found an efficiency to be 17.3 percent for one described run using blood as a substrate. This corresponds closely with the percent efficiency secured in our tests for the protease of midguts dissected one hour after feeding.

The highest percent efficiency secured here was apparently 338 percent at 18 hours after feeding. This abnormally high figure may be due to the abnormal enzyme:substrate ratio since more than seven times as much protein was added to the enzyme solution than necessary for maximum digestion. Activity *in vivo* may be limited by the volume of blood the midgut is capable of holding. The high figure may be due to incorrect assumptions, e.g. it is likely that the complete digestion of the blood by a living mosquito requires nearer 48 than 72 hours.

SUMMARY

1. Immediately following a blood meal the protease activity of the midgut of *Aedes aegypti* rapidly drops in 5 minutes to below the residual level characteristic of the unfed mosquito. This may be due to an antitrypsin in the blood or to depletion of the enzyme by the substrate.

2. The drop is followed by an equally rapid increase for the first few hours. The rate of increase gradually falls off but the amount of activity continues to increase until a maximum 26-fold increase is evident at 18 hours. By 48 hours activity is greatly reduced but still above the residual value.

3. In contrast, sugar solution feeding produces an initial two-fold rise in protease activity during the first hour, but by two hours the activity has dropped again to the residual value.

4. These data contrast with those of other authors for other insects in which brief feeding caused an increase in the activity of all digestive enzymes, irrespective of the kind of food taken.

5. The feeding experiments favor either a hormonal or a secretagogue hypothesis of enzyme stimulation, but our injection experiments favor the latter hypothesis.

6. The percent efficiency of substrate hydrolyzed in our tests compared to the estimated rate of a living mosquito digesting a blood meal agrees fairly well with the figure given by Fisk (1950) for one hour after feeding. However our maximum rate (at 18 hours) corresponds to 338 percent efficiency, an unrealistic value.

LITERATURE CITED

- Beiderman, W.** 1898. Beiträge zur vergleichenden Physiologie der Verdauung I. Die Verdauung der Larvae von *Tenebrio molitor*. Arch. ges. Physiol., 72: 105-62.
- Charney, J.,** and **R. M. Tomarelli.** 1947. A colorimetric method for the determination of proteolytic activity of duodenal juice. Jour. Biol. Chem., 171: 501-5.
- Day, M. F.,** and **R. F. Powning.** 1949. A study of the processes of digestion in certain insects. Austral. Jour. Sci. Res. (series B), 2: 175-215.
- Fisk, F. W.** 1949. Studies on digestive enzymes in certain mosquitoes. Ph.D. dissertation, U. of Minn.
- . 1950. Studies on proteolytic digestion in adult *Aedes aegypti* mosquitoes. Ann. Ent. Soc. Amer., 43: 555-72.
- Hawk, P. B.; B. L. Oser;** and **W. H. Summerson.** 1947. Practical physiological chemistry. 12th Ed. 1323 pp. Blakiston Co., Philadelphia, Pa.
- Scheer, B. T.** 1948. Comparative physiology. 563 pp. John Wiley and Sons, New York.
- Schlottke, E.** 1937a. Untersuchungen über die Verdauungsfermente von Insecten. I. Die Verteilung der Fermente in Darm Kanal von Flieschfressenden Carabiden and die Änderungen ihrer Konzentration während der Verdauung. Zeitschr. Vergl. Physiol., 24: 210-47.
- . 1937b. Untersuchungen über die Verdauungsfermente von Insecten. II. Die Fermente der Laub-und Feldheuschrecken und ihre Abhängigkeit von der Lebensweise. Zeitschr. Vergl. Physiol., 24: 422-50.
- . 1937c. Untersuchungen über die Verdauungsfermente von Insecten. III. Die Abhängigkeit des Fermentgehaltes von der Art der Nahrung. Versuche an *Periplaneta orientalis* L. Zeitschr. Vergl. Physiol., 24: 463-92.
- Schmitz, A.** 1937. Über die Freilung von Activen Trypsin aus Blut Plasma. Zeitschr. Vergl. Physiol. Chem., 250: 37-46.
- Shambaugh, G. F.** 1951. Protelytic enzyme stimulation and activity in relation to time after feeding in *Aedes aegypti* Linn. mosquitoes. Masters Thesis, Ohio State University.
- Summer, J. B.,** and **G. F. Somers.** 1947. Chemistry and methods of enzymes. 2nd Ed. 415 pp. Academic Press, Inc. New York.
- Tomarelli, R. M.; J. Charney;** and **M. L. Harding.** 1949. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. Jour. Lab. and Clin. Med., 24: 428-33.
- Wigglesworth, V. B.** 1947. Insect physiology. 434 pp. E. P. Dutton & Co. New York.
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