# THE DIGESTIVE ENZYMES OF THE STABLE FLY, *STOMOXYS CALCITRANS* (L.).[1]

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In recent years, an ever-increasing number of publications dealing with the digestive physiology of blood-sucking insect pests have appeared. The greater part of these studies have dealt with digestion in mosquitoes, such as *Aedes aegypti* and *Anopheles quadrimaculatus*, which are known to be vectors of etiological organisms in diseases affecting man.

In this study the stable fly was chosen as a test insect for several reasons, chiefly the relative ease with which large numbers can be reared for experimental purposes. In this insect, both sexes feed upon blood, and do so readily under laboratory conditions. While the stable fly has not been definitely incriminated as a vector of the etiological organism of any human disease, it remains a pest of considerable economic importance due to the vicious bites it inflicts upon man and other animals, particularly livestock.

*Rearing and Feeding the Flies.* The procedure followed in rearing the insects has already been published (Champlain, *et. al.*, 1954). All blood used for feeding flies was bovine, and contained 250 ml. of 5 percent sodium citrate solution per gallon in order to prevent coagulation.

Flies used in enzyme tests were allowed to feed to repletion upon blood poured over a piece of cotton covering the bottom of a polystyrene plastic dish measuring about 4 in. in diameter and 1 in. in depth. Before it was placed in a cage containing flies, the feeding dish was placed in a pan of warm water until the blood felt tepid to the fingertips. When this was done, flies which had been deprived of both food and water for a 24-hour period began to feed almost immediately.

Experiments indicated that the flies fed most readily when they were about 4 days old. A routine feeding procedure was adopted in which flies were allowed to emerge in a holding cage which was provided with a plastic dish containing cotton soaked with 5 percent sucrose solution as food. At the end of two days, the food dish was removed for a 24-hour period, following which the blood meal mentioned above was placed in the cage.

*Equipment and Care of Glassware.* In all colorimetric procedures, a Klett-Summerson Model 800–3 photoelectric colorimeter was used. Before readings were made, the machine was adjusted to read zero when a Klett tube containing 5 ml. of distilled water was put in position. A "Magni-Whirl" Model MW1110 water bath operating at $40 \pm 0.5°$ C was used to incubate test mixtures.

For centrifuging, a Sorvall angle centrifuge was used. The rheostat position required to produce a speed of 5500 r.p.m. was determined by means of a stroboscope, and this setting was used exclusively in enzyme runs.

All glassware coming in contact with enzymatic or other proteinaceous material was scrubbed thoroughly with a solution of "*Dreft*" detergent, and then was rinsed several times with tap water. The glassware was then immersed in concentrated nitric acid and allowed to remain there overnight. Three rinses with tap water followed its removal from the acid, and these were followed by four rinses with distilled water. After draining, a rinse of 95 percent ethyl alcohol, followed by

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one of acetone, removed the water. Complete volatilization of the acetone was insured by placing the glassware in an oven operating at 100° C. for about 2 hr.

*Qualitative Tests.* Since a review of the literature did not disclose any previous work on stable fly digestive enzymes, gross tests were made in order to establish the presence or absence of the various enzymes. If a test was positive, various portions of the alimentary tract, including accessory structures, were tested separately in order to localize the site of secretion. If an enzyme was found to be present in the midgut, but absent in portions of the alimentary tract lying anterior to it, the inference was made that it actually was secreted in the midgut. If the enzyme was present in a structure anterior to the midgut, and also in the midgut, it was inferred that the anterior region was actually the site of secretion and that the enzyme was present in the midgut due to the passage of materials through the alimentary canal. Only one enzyme, a weak salivary amylase, was found in structures anterior to the midgut. All other digestive enzymes encountered in the midgut region appeared to be secreted there.

Due to limitations of space, the results of these qualitative tests will be presented in tabular form (table 3). Detailed descriptions of some of the tests have been omitted, but the type of each test has been indicated. Most of the tests were techniques which have been used for some time and reference to almost any laboratory manual of insect physiology will provide the necessary details. The tests used in proteolytic enzyme experiments, however, are less commonly used, and detailed descriptions of them follow.

*Tests for Pepsin-like Enzyme.* In the past, tests for pepsin have often been omitted, with a statement that this type of enzyme was not found in insects. However, Greenberg and Paretsky (1955) provide some evidence for the presence of such an enzyme in the house fly. For the sake of completeness, tests were run, using stable flies as an enzyme source.

The substrate, sulfanilic acid-azoalbumin, was prepared according to the method of Tomarelli, Charney, and Harding (1949). Since pepsin-like enzymes are most active in the pH range from about 1.5 to 3.0, separate substrate solutions were made up covering this range by 0.5 pH units. A given solution was prepared by dissolving 250 mg. of azoprotein in about 5 ml. of distilled water. Next, the micro beaker containing the dissolved material was placed on a Beckman Model H–2 pH meter fitted with micro electrodes. The pH of the solution was adjusted to the desired level by dropwise addition of 0.2M HCl, and then the liquid was transferred to a graduated glass cylinder and its final volume was brought to 10 ml. by the addition of a suitable volume of buffer solution of the same pH. This procedure was repeated until individual substrate solutions had been prepared for pH 1.5, 2.0, 2.5, and 3.0. Each solution contained 25 mg. azoprotein/ml.

Chromophoric protein derivatives, such as azoalbumin and azocasein, are formed by the coupling of a diazotized aryl amine with a protein 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 fragments which are soluble in trichloroacetic acid (Charney and Tomarelli, 1947). The appearance of color in the trichloroacetic acid filtrate depends upon an intact diazotized amino acid or peptide fragment. The intensity of color in the filtrate following protease activity is a direct function of the proteolytic activity of the enzyme source and its measurement serves as the basis of this technique.

Whole stable fly bodies were used as an enzyme source in order that the enzyme would not be overlooked if it was present somewhere outside of the alimentary tract. The bodies of 16 flies which had been digesting a blood meal for 4 hr. were ground up in a mortar containing a little clean sand and 1 ml. of pH 1.5 buffer solution. The resulting brei was extracted with an additional 3 ml. of buffer solution. The mixture was transferred to a centrifuge tube and centrifuged

at 4500 r.p.m. for 5 min. in order to clear the debris. Aliquots consisting of 0.5 ml. of the centrifuged extract were then placed in each of six micro test tubes, and stored temporarily in the deep freezer. A similar procedure was followed in preparing breis for pH values of 2.0 and 2.5. In the preparation of brei at pH 3.0, double quantities were used and aliquots placed in 12 micro tubes, six of which were boiled 5 min. prior to storage in the deep freezer, and severed as blanks.

When all breis had been prepared, they were removed from the deep freezer and allowed to thaw. Next, 0.5 ml. of buffer solution of the same pH in which the brei had been extracted was added, along with 0.25 ml. of azoalbumin substrate solution at the same pH. All tubes were then placed in the water bath for 4 hr. at 40° C. At the end of this time, all tubes were removed from the water bath and were given what will subsequently be referred to as "the routine protein test". The contents of the micro tubes were poured into numbered centrifuge tubes which contained 1.5 ml. of 10 percent trichloroacetic acid. Each micro tube was then rinsed with an additional 1 ml. of trichloroacetic acid, and this rinse was added to the contents of the appropriate centrifuge tube. The precipitated protein was separated by centrifuging for 10 min. at 5500 r.p.m. Next, 2.5 ml. of the

TABLE 1

*Colorimeter readings obtained in protease tests performed on whole bodies of flies*

| SUBSTRATE | pH | HOURS INCUBATED | LOWEST READING | HIGHEST READING | AVERAGE | CORRECTED AVERAGE |
|---|---|---|---|---|---|---|
| azoalbumin | 1.5 | 4 | 19 | 21 | 19.7 | — |
| " | 2.0 | 4 | 18 | 23 | 20.5 | — |
| " | 2.5 | 4 | 19 | 22 | 20.8 | — |
| " | 3.0 | 4 | 27 | 30 | 28.0 | — |
| " | blank (3.0) | 4 | 28 | 31 | 29.3 | 0 |
| azocasein | 7.8 | 0 | 30 | 35 | 34 | 6 |
| " | 7.8 | 2 | 58 | 65 | 62 | 34 |
| " | 7.8 | 4 | 109 | 122 | 115 | 87 |
| " | 7.8 | 6 | 166 | 178 | 171 | 143 |
| " | blank (7.8) | 6 | 27 | 29 | 28 | 0 |

supernatant liquid from each tube were placed in a calibrated Klett colorimeter tube, and then 2.5 ml. of 0.5N sodium hydroxide solution were added to each Klett tube. The addition of the sodium hydroxide solution caused a shift in pH and served to intensify the color. Readings were determined by means of the colorimeter, using the 440 $m\mu$ blue filter.

The amount or the condition of the blood contained in the digestive tracts of the flies had no effect upon the results of the tests since the blood proteins would be precipitated by the trichloroacetic acid, and any colored materials soluble in this acid would be equal in both blanks and runs. The results of these tests are given in table 1. The data show that the readings of all tubes were very low, none exceeding the blank level. Since there was no significant increase in activity in the active tubes, the results were interpreted as being negative for a pepsin-like enzyme.

*Tests for Trypsin-like Enzyme.* A second chromophoric protein derivative, sulfanilamide-azocasein, was prepared according to the method of Charney and Tomarelli (1947), and their directions were followed in preparing a stock solution. The basis for the technique and the test methods are similar to those used in testing for pepsin, except that pH values in the alkaline range were used.

At the time these tests were made, the pH optimum for the hydrolysis of azocasein by stable fly trypsin was not known, but tests were run using buffer solutions of pH 7.8, since the pH optima of many insect trypsins lie near this value.

The bodies of 44 flies which had been digesting a blood meal for 4 hr. were ground up in a mortar containing a little clean sand and 1 ml. of pH 7.8 buffer solution. The brei was extracted with an additional 10 ml. of buffer solution, and 0.5 ml. aliquots were withdrawn and placed in each of 20 micro tubes. Four of these tubes were boiled for 5 min., serving as blanks. Next, 0.5 ml. of pH 7.8 buffer solution was added to each of the 20 micro tubes, followed by the addition of 0.25 ml. of azocasein stock solution. Four of the active tubes were precipitated immediately by the addition of 1.5 ml. of 10 percent trichloroacetic acid and then tested by means of the routine protein test. The remaining 12 active tubes and the 4 blanks were placed in the water bath operating at 40° C. At the end of 2 hr., 4 hr., and 6 hr., four active tubes were withdrawn and tested; the blanks were tested at the end of 6 hr.

A similar brei was prepared and a similar arrangement of micro tubes was used on the following day, providing 8 replicates.

Results of these tests are given in table 1. The corrected average was obtained by subtracting the average blank reading from the various average readings. These data show a marked increase in optical density as time increases, indicating increasing hydrolysis of the chromophoric protein substrate, and the test was interpreted as being positive for trypsin. A statistical analysis of the data, using

TABLE 2

*Synopsis of qualitative enzyme tests*

| ENZYME | TEST USED | RESULT | SITE OF SECRETION |
|---|---|---|---|
| Invertase | Benedict test for reducing sugars. | Positive | Midgut |
| Lactase | Phenylhydrazine test for reducing sugars. | Negative | — |
| Lipase | Change in pH of alkaline substrate-brei mixture. Bromthymol blue indicator used. | Positive | Midgut |
| Amylase | Non-appearance of blue color in starch substrate when tested with iodine indicator. | Positive (weak) | Salivary glands |
| Pepsin-like proteinase | Hydrolysis of azoalbumin substrate. | Negative | — |
| Trypsin-like proteinase | Hydrolysis of azocasein substrate. | Positive | Midgut |

a t-test of a regression coeffiicient, showed that the rate of change differed signficantly from zero at a 2 percent confidence level. The calculated "t" value was 9.889.

Similar tests run upon separate portions of the gut produced substantially the same results when the midgut was tested.

In testing for lactase activity, the results were interpreted as being negative even though precipitates formed in all tubes, including blanks and controls, when they were given the osazone test for reducing sugars. These precipitates were identified as glucosazone and lactosazone. Precipitates also formed when phenylhydrazine reagent was added to aliquots of midgut brei to which no substrate had been added. This phenomenon may have been due to the normal glucose level of the blood ingested by the flies. Final interpretation was based upon the fact that microscopic examination of the osazone precipitates failed to reveal crystals of galactosazone, a product which should have appeared if the reducing sugars had been end-products of lactase activity. Table 2 is a synopsis of qualitative tests.

## QUANTITATIVE PROTEASE TESTS AND RESULTS

*pH Optima for Stable Fly Trypsin.* Earlier authors (Baldwin, 1949; Northrop, 1939) have pointed out that an optimum pH is characteristic of a given enzyme, though under certain special conditions and in certain groups of enzymes the pH optimum may vary. This is true of the proteolytic enzymes, different optima being found with different protein substrates. Therefore, the next group of tests were run in order to determine the pH optimum of fly trypsin in the hydrolysis of azocasein and azoalbumin, respectively.

*pH Optimum, Tryptic Hydrolysis of Azoalbumin.* Since trypsin-like proteinases generally are most active at alkaline pH values between 7 and 10, preliminary experiments, using azoalbumin as a substrate, were run covering this pH range.

Individual substrate solutions were made up for each pH value tested by dissolving 250 mg. of azoalbumin in 5 ml. of distilled water, and then bringing the pH of the solution to the desired point by dropwise addition of 0.2M sodium hydroxide solution, followed by dilution to a final volume of 10 ml. by the addition of an appropriate volume of buffer solution of that pH. This procedure produced solutions containing 25 mg. azoprotein/ml.

TABLE 3

*Colorimeter readings obtained in determination of pH optimum*

| SUBSTRATE | pH | LOWEST READING | HIGHEST READING | AVERAGE OF 8 | CORRECTED AVERAGE |
|---|---|---|---|---|---|
| azoalbumin | 7.7 | 198 | 200 | 199.5 | 176.5 |
| " | 7.8 | 206 | 209 | 208 | 185 |
| " | 7.9 | 213 | 215 | 214.5 | 191.5 |
| " | 8.0 | 208 | 210 | 209 | 186 |
| " | 8.1 | 200 | 201 | 200.5 | 177.5 |
| " | blank (8.1) | 22 | 24 | 23 | 0. |
| azocasein | 7.6 | 248 | 251 | 250 | 227 |
| " | 7.7 | 251 | 253 | 252 | 229 |
| " | 7.8 | 256 | 258 | 256.8 | 233.8 |
| " | 7.9 | 248 | 250 | 249 | 226 |
| " | 8.0 | 245 | 249 | 248 | 225 |
| " | blank (8.0) | 22 | 25 | 23 | 0 |

Results of these tests indicated a maximum of activity occurring at about pH 8. Therefore, the range lying between pH 7 and 9 was tested, being covered by 0.2 pH units. These tests verified the results obtained in the first runs, almost equal values being obtained at pH 7.8 and 8.0.

Final tests were made at pH values 7.7, 7.8, 7.9, 8.0, and 8.1. The preparation of the brei was altered somewhat in order to minimize random error due to variations in separate breis by following a procedure which would produce quite uniform aliquots. The midguts of 60 flies which had been digesting a blood meal for 4 hr. were dissected out in small groups which were stored in the deep freezer until all had been accumulated. Following thawing, they were ground up and transferred to a small graduated glass cylinder. The final volume was brought to 3 ml. by the addition of an appropriate volume of distilled water. Use of aliquots of 0.1 ml. provided enzymatic material equivalent to that contained in 2 midguts.

Aliquots of brei were placed in each of 24 micro tubes. These tubes were arranged in groups of four each, each group representing a pH value. pH 8.1 was represented by eight tubes. Next, 0.9 ml. of buffer solution of the desired pH was placed in the corresponding tubes. At this point, four of the pH 8.1 tubes were boiled for 5 min., serving as blanks. Then 0.25 ml. of azoalbumin substrate

solution of the appropriate pH was placed in each corresponding micro tube. All tubes were incubated in the water bath at 40° C. for 4 hr. At the end of this time, they were removed and given the routine protein test. The runs were repeated, with a total of eight replicates, the results of which are given in table 3 and figure 1.

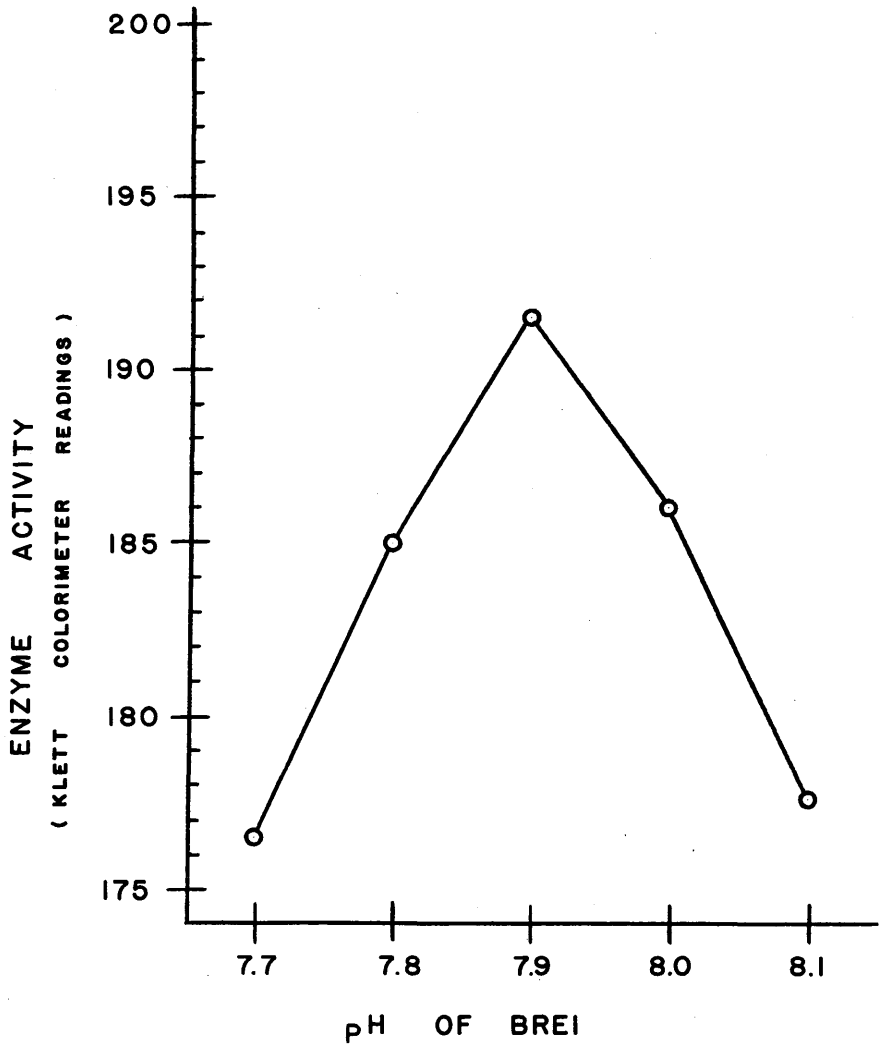


FIGURE 1. pH optimum of stable fly protease with azoalbumin as substrate. *Brei:* midguts of adult flies, both sexes, 4 hr. after a blood meal. *Incubation:* 4 hr. at 40° C at pH values shown.

These results show a maximum of activity occurring at pH 7.9, indicating that this value represents the optimum pH for the hydrolysis of azoalbumin by stable fly trypsin under the conditions of the experiment.

*pH Optimum, Tryptic Hydrolysis of Azocasein.* The procedure used in these tests was identical to that just described for determining the pH optimum for hydrolysis of azoalbumin.

Preliminary experiments indicated a maximum of activity at about pH 7.8. Therefore, final runs were made using the pH values 7.6, 7.7, 7.8, 7.9, and 8.0. Blanks were run at pH 8.0, and the routine protein test was employed. Eight replicates were used. Results are given in table 3 and figure 2.

These data show a maximum of activity occurring at pH 7.8, indicating that this value represents the optimum pH for the hydrolysis of azocasein by stable fly trypsin under the conditions of the experiment.

*Protease Activity Related to Time.* The phenomenon of an increase in enzymatic activity following feeding has been known for over half a century, Biedermann (1898) being one of the earliest writers to mention its occurrence. Fisk and Shambaugh (1952) note such an increase in female *Aedes* mosquitoes. They also note an initial drop in protease activity immediately following feeding, a change which was not detected by Fisk (1950). Fisk and Shambaugh (1952) also

TABLE 4

*Colorimeter readings obtained in testing relationship of Protease activity to time of digestion of a blood meal*

| TIME OF DIGESTION | LOWEST READING | HIGHEST READING | AVERAGE | CORRECTED AVERAGE |
|---|---|---|---|---|
| 0 | 39 | 39 | 39 | 19 |
| 5 min. | 39 | 41 | 40 | 20 |
| 15 min. | 50 | 53 | 52 | 32 |
| 30 min. | 60 | 62 | 61 | 41 |
| 1 hr. | 86 | 91 | 89 | 69 |
| 2 hr. | 102 | 107 | 105.5 | 85.5 |
| 3 hr. | 177 | 183 | 180.5 | 160.5 |
| 6 hr. | 283 | 292 | 287.5 | 267.5 |
| 9 hr. | 335 | 341 | 339.2 | 319.2 |
| 12 hr. | 355 | 366 | 360.5 | 340.5 |
| 13 hr. | 386 | 392 | 388 | 368 |
| 14 hr. | 383 | 391 | 386 | 366 |
| 15 hr. | 363 | 369 | 365.2 | 345.2 |
| 18 hr. | 330 | 336 | 334 | 314 |
| 24 hr. | 303 | 310 | 306 | 286 |
| blank | 19 | 21 | 20 | 0 |

demonstrated that protease activity in the female mosquito, *Aedes aegypti*, is at its maximum about 18 hr. after a blood meal, after which time the protease activity begins to diminish. The subsequent experiments were designed in order to determine how much time intervened between feeding and maximum proteolytic activity in the stable fly.

Preliminary tests indicated a maximum of enzyme activity at about 12–14 hr. after feeding.

Flies fed upon citrated bovine blood were captured in an aspirator, and groups of 20 were allowed to digest the meal for the time periods given in the table of results. When this period had elapsed, they were killed and the midguts were dissected out. Since a large number of flies was used, and since different groups represented different digestion intervals, dissection was performed by group, and the breis were held frozen at $-15°$ C until all had been prepared. Twenty fly guts were used in each brei, diluted to a final volume of 1 ml. (Use of 0.1 ml. aliquots provided the previously-adopted standard of 2 midguts per aliquot.) Azocasein stock solution at pH 7.8 and buffer solution of the same value were used.

Aliquots of 0.1 ml. of each brei were placed in each of two micro tubes. Nine-tenths ml. of pH 7.8 buffer solution was added to each tube. At this point, two tubes were boiled for 5 min., serving as blanks. (Four zero-hour tubes were set

up, two being boiled and two being retained as active). Then 0.25 ml. of azocasein stock solution was added to each micro tube, and all tubes incubated in the water bath at 40° C for 4 hr. At the end of this time, they were given the routine protein test. Similar tests, run on four different days, provided 8 replicates. Results are given in table 4 and figure 3.

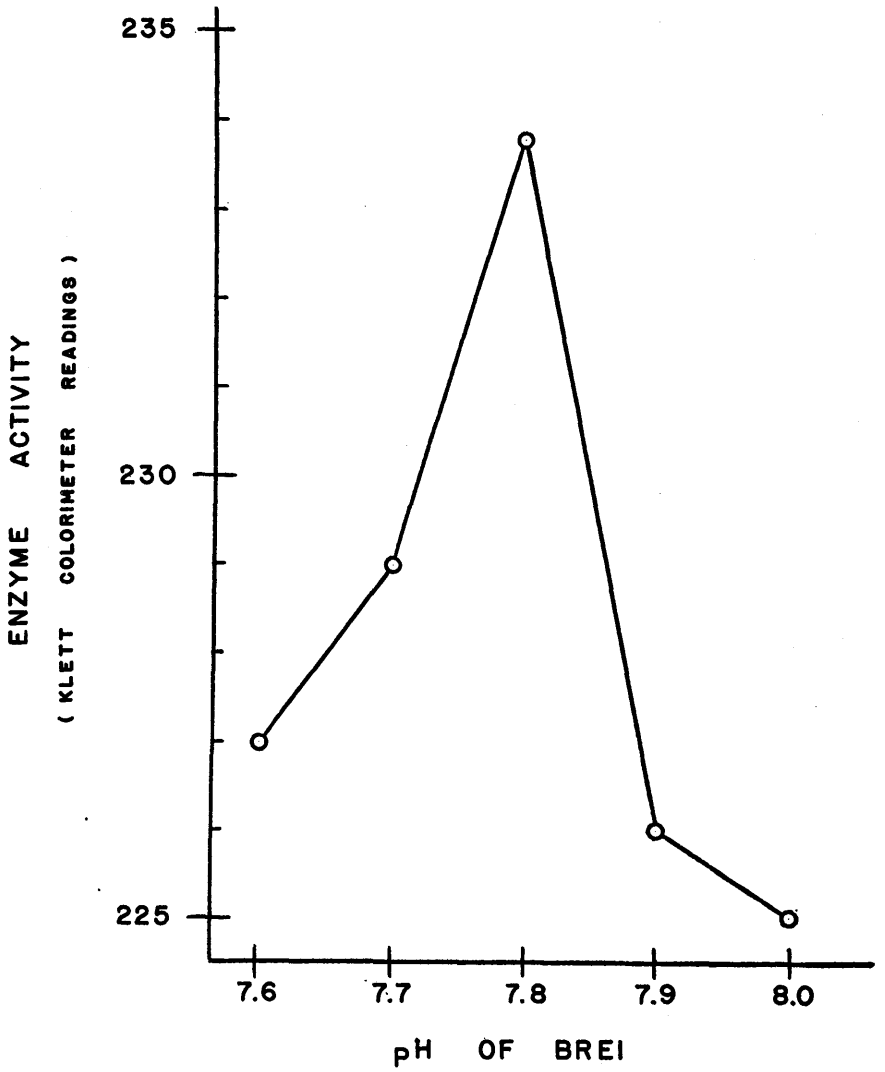


FIGURE 2. pH optimum of stable fly protease with azocasein as substrate. *Brei:* midguts of adult flies, both sexes, 4 hr. after a blood meal. *Incubation:* 4 hr. at 40° C at pH values shown.

These results showed a sharp rise in activity after the first 5 min. This rise continued for about 6 hr., at which time the rate decreased somewhat. Maximum proteolytic activity appeared to occur about 13 hr. after a blood meal. The activity began to decline after the maximum was reached and continued to drop during the remainder of the time interval covered in the test.

As noted above, Fisk and Shambaugh (1952), working with *Aedes aegypti* females, observed a significant decrease in proteolytic activity in the first 5 min. following a blood meal. The results of the tests under discussion do not show a similar phenomenon in the stable fly. The readings obtained at 5 min. are approximately the same as those obtained at zero hours. The last-named authors attribute their lowered 5-minute readings to a possible "depletion" of the enzyme by its substrate. It is worth noting, however, that a comparison is being made between two insects in which ingested blood is stored in quite different ways. It has been known for a long time that when a mosquito ingests blood, this liquid passes directly to the stomach, while other fluids, such as nectar, go first to the crop and the dorsal diverticula. In the muscoid bloodsuckers, such as *Glossina* and *Stomoxys*, ingested blood passes into the crop, where no digestion or denaturation occurs. The crop functions simply as a storage organ, and quantities of blood are released from it periodically, this blood entering the midgut.

This divergence being the case, variation in phenomena related to blood feeding logically might be expected. Phenomena related to feeding upon nectar or sucrose solution should be in more general agreement, because the relative mechanisms are more similar.

TABLE 5

*Colorimeter readings obtained in tests for protease activity in sucrose-fed stable flies*

| TIME OF DIGESTION | LOWEST READING | HIGHEST READING | AVERAGE | CORRECTED AVERAGE |
|---|---|---|---|---|
| Unfed | 42 | 45 | 43 | 21 |
| 5 min. | 42 | 45 | 43.2 | 21.2 |
| 30 min. | 44 | 46 | 45 | 23 |
| 1 hr. | 54 | 58 | 56.2 | 34.2 |
| 2 hr. | 49 | 51 | 50 | 28 |
| 4 hr. | 43 | 46 | 45.7 | 23.7 |
| 8 hr. | 45 | 47 | 46.2 | 24.2 |
| 16 hr. | 45 | 47 | 46 | 24 |
| 24 hr. | 42 | 45 | 44.3 | 22.3 |
| blank | 21 | 23 | 22 | 0 |

The proteolytic enzymes tested are localized in the midgut in both the mosquito and the stable fly. Currently-accepted theories of the formation of an enzyme-substrate complex imply a rather intimate combination between molecules of the enzyme and the substrate, and in the case of the mosquito, the sudden admission of a large volume of blood substrate directly into the region where the enzymes are localized conceivably could produce the "depletion effect". On the other hand, the mechanism in the stable fly regulating admission of blood to the midgut is such that only a comparatively small part of the total volume of ingested blood would reach the midgut in the initial 5-minute period. This relatively small amount of substrate might not be sufficient to produce a noticeable "depletion effect", especially in view of the fact that enzyme secretion might increase even in this short time, to the point where it would counterbalance any small initial depletion. In any event, no significant drop in proteolytic activity 5 min. after a blood meal was observed in these experiments on the stable fly.

The experimental establishment of a maximum of protease activity at about 13 hr. apparently would contradict the statement of Metcalf and Flint (1951) that "a single stable fly feeds several times a day, taking a drop or two of blood each time". On the basis of observed results, it would seem that two feedings per day would be a maximum. At the end of 24 hr., a very small amount of blood,

which is dark in color and almost rubbery in consistency, remains in the gut. Under laboratory conditions, absolute digestion of a blood meal would require a little more than 24 hr.

It should be noted that the statement of Metcalf and Flint, mentioned above, pertains to stable flies living under natural conditions, while the observations made here pertain to flies living under laboratory conditions. Wild flies undoubtedly are more active than flies in laboratory cages, and probably digestion proceeds more rapidly in the former. In addition, Fisk (1950) observed that the physical nature of the ingested blood may be a factor in digestive efficiency. All blood used in the laboratory was treated with sodium citrate to prevent coagulation, and this may slow down the overall digestive rate, especially if coagulation is a normal digestive phenomenon, which would appear to be the case in the stable fly, as

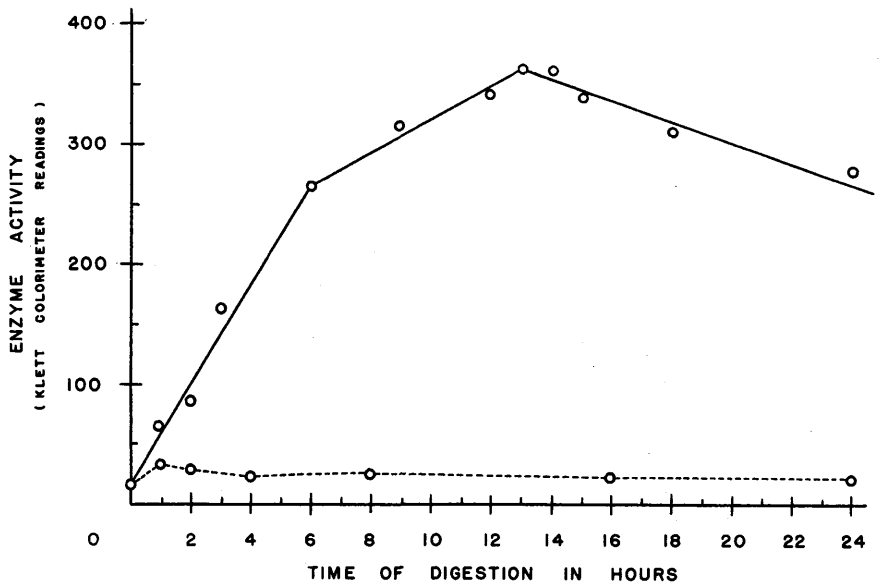


FIGURE 3. Protease activity at various intervals after feeding. (Solid line—blood-fed series; broken line—sucrose-fed series.) *Brei:* midguts of adult flies, both sexes. *Substrate:* azocasein. *Incubation:* 4 hr. at 40° C and pH 7.8.

indicated by the rubbery consistency of the blood remaining in the gut at the end of 24 hr. Some insects secrete salivary anti-coagulins which would tend to neutralize the discrepancy just mentioned, but according to Cornwall and Patton (1914), such a salivary anti-coagulin is absent in *Stomoxys*.

In view of the different environments occupied by wild flies and laboratory flies, as well as the differing foods they ingest, the statement of Metcalf and Flint may not be incorrect.

*Protease Activity as Related to Food.* It was originally planned that a similar series of tests would be run using flies which had fed upon skimmed milk. All attempts to obtain predictable feeding responses failed completely when this material was offered as food.

Since the ingestion of food stimulates the production of all enzymes normally found, rather than one specific enzyme, tests were included to measure the effect of the ingestion of a non-proteinaceous food, sucrose, on protease activity.

Flies were allowed to digest a meal of 5 percent sucrose solution for various intervals as set forth in table 5. Breis were made up and tested on three different

days as described in the preceding section relating to time, providing six replicates. Results are given in table 5 and figure 3.

These data show a slight initial increase in protease activity during the first hour, after which the activity declines and remains close to the value obtained using unfed flies. The general pattern of activity is similar to that obtained by Fisk and Shambaugh (1952), although they cite 30 min. as the time at which peak activity occurred. They do not present data for 1-hour and 2-hour insects. According to table 5, maximim protease activity occurs in the midgut of the stable fly about 1 hr. after a meal of sucrose solution.

## SUMMARY

Qualitative tests were positive for trypsin, invertase, lipase, and amylase. Tests for pepsin and lactase were negative.

A pH optimum for the hydrolysis of sulfanilic acid-azoalbumin by stable fly trypsin was determined as being pH 7.9.

A pH optimum for the hydrolysis of sulfanilamide-azocasein by stable fly trypsin was determined as being pH 7.8.

When protease activity was related to time of digestion of a blood meal, maximum activity was obtained at about 13 hr.

Protease activity following a meal of sucrose solution showed a slight increase for about an hour, but this activity quickly declined and remained close to the residual value.

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