

# A STUDY OF THE TRYPSINLIKE PROTEASE OF THE ADULT STABLE FLY, *STOMOXYS CALCITRANS* (L.)<sup>1</sup>

ROBERT A. PATTERSON<sup>2</sup> AND FRANK W. FISK

*Department of Zoology and Entomology, The Ohio State University, Columbus*

## INTRODUCTION

For the past few years a study of the digestive mechanisms of the adult stable fly, *Stomoxys calcitrans* (L.), has been under investigation in this laboratory. The initial work, which dealt with the qualitative identification of the midgut digestive enzymes and the quantitative stimulation of protease by feeding has been reported previously (Champlain and Fisk, 1956). The present study is in part a continuation of Dr. Champlain's work on the qualitative characterization of the trypsinlike activity of the stable fly and in part an attempt to purify the trypsinlike component of the midgut brei. Purification was attempted by fractionation methods as well as by zone electrophoresis on paper and on starch gels.

## TECHNIQUES AND EQUIPMENT

*Rearing of the stable flies.*—Stable flies were reared according to the method of Champlain, Fisk, and Dowdy (1954). Pupae were taken from the breeding culture and held in a refrigerator for a week to ten days as needed. Flies for laboratory use were reared from these pupae in small rearing cages. After emergence, the adults were maintained on a ten percent sucrose diet for three days. Then the sugar solution was removed and the adults were starved for 24 hours. A meal of fresh whole citrated bovine blood was fed to the flies from a blood soaked cotton pad. After a given time interval, usually 12 hours, the cage was placed in a deep freezer. When the flies were immobilized by cooling, they were transferred to small plastic cups which were partially filled with ice and water. Midguts were dissected from these flies. The 12 hour period following feeding was shown by Champlain (1955) to coincide with the interval of maximal proteolytic activity in the midgut. Following dissection, the midguts were homogenized in buffer at pH 7.8 and stored in a deep freezer.

*Enzyme activity.*—The trypsinlike activity was determined by an adaption of the method of Charney and Tomarelli (1947) using a sulfanilamide azocasein substrate dissolved in Sigma "7-9" *Tris* buffer (2 amino-2-hydroxymethyl-1, 3-propanediol) (Sigma Chemical Company, St. Louis, Missouri) adjusted to pH 7.8. The azocasein substrate was used in the reaction mixture at a concentration of ten mg per ml. The homogenate-substrate reaction mixtures were incubated in a water bath at 40°C. A zero time and a final time determination were made on this reaction mixture. This consisted of treating one ml aliquots of the reaction mixture with two ml of ten percent trichloroacetic acid. This precipitated the unhydrolyzed protein. After centrifuging, two ml of the supernatant were removed and added to three ml of 0.5 N sodium hydroxide. Alkalinization intensified the color of the solution. A Klett-Summerson Photoelectric Colorimeter, equipped with a 420 millimicron filter, was used to measure the amount of light transmitted by this solution. A water blank was treated in the same manner to determine the amount of chromophore released from the substrate nonenzymatically. Usually this was negligible.

The activity of the reaction mixture was determined by correcting the final

<sup>1</sup>Part of a dissertation submitted by the senior author in partial fulfillment of the degree Doctor of Philosophy.

<sup>2</sup>Present address: Department of Zoology, Arizona State College, Tempe, Arizona.

Klett reading to compensate for the nonenzymatic hydrolysis and the initial amount of hydrolysis products present in the solution. Activity could be reported in terms of Klett units per ml of homogenate by appropriate dilution factors. In a few cases activity is reported in terms of mg of substrate hydrolyzed. This was determined by use of a standard curve based on the alkalization of serial dilutions of the substrate. When the substrate is made alkaline, the color density of the solution is the same as if the substrate had been completely hydrolyzed and the soluble chromophore made alkaline for colorimetric analysis.

A second substrate described by Tomarelli, Charney, and Harding (1949) was used in the same manner. This substrate, sulfanilic acid azoalbumin, was used in the reaction mixture at a concentration of 12.5 mg per ml.

*Paper electrophoresis.*—A *Misco* Paper Electrophoresis Apparatus (Microchemical Specialties Company, Berkeley, California) with a D. C. Power supply (Biophysical Instruments Company, supplied by A. H. Thomas Company, Philadelphia, Pennsylvania) was used to study the electrophoretic mobility of the midgut homogenate. Whatman No. 1 filter paper, supplied in a roll one and one half inches wide, was cut into 14 inch long strips and placed on the tray. The ends of the paper were allowed to dip into the buffer in the end cells. The homogenate was applied with a *Spinco* Applicator (Specialized Instruments Corporation, Division of Beckman Instrument Company, Inc., Belmont, California) on a pencilled line across the center of each strip. The electrophoresis apparatus was placed in a refrigerator held at 8°C in order to avoid over-heating the protein materials due to the resistance of the paper to the applied voltage.

*Starch gel electrophoresis.*—Plastic trays were constructed from *Plexiglas* to have dimensions of 30 by 5 by 2 cm. A glass plate was cut to serve as a cover. The trays were filled with starch gel and connected to the end cells of the *Misco* paper electrophoresis apparatus by means of filter paper wicks. Both *Tris*-citrate and barbital buffers were used in the formation of the starch gels.

The starch gel was prepared from commercial corn starch. A slurry of *Cream* corn starch (Staley Manufacturing Company, Decatur, Illinois), 10 gm in 20 ml distilled water, was added to 100 ml of boiling *Tris*-citrate buffer. Six gm of *Super Cel* and 80 ml of distilled water were added. This mixture was allowed to boil for ten minutes while being continually stirred. The final molarity of the buffer in the gel was 0.03. After the gel had cooled slightly, it was poured into the plastic trays. Then they were covered with the glass plate and placed in the refrigerator overnight.

*Protein determinations.*—Both the Hengar Micro-Kjeldahl (Hengar Company, Philadelphia, Pennsylvania) and the Folin-Ciocalteu protein determination (Lowry et. al., 1951) methods were used to check the protein nitrogen and the protein content of the homogenates. Armour bovine albumin (Fraction V) served as the empirical standard for the Folin-Ciocalteu determination.

#### EXPERIMENTAL

*Comparisons of activity of homogenates from male and female stable flies.*—Male and female flies were separated following feeding with citrated bovine blood. The flies were dissected ten hours later, and the trypsinlike activity of the separate homogenates was determined. In these experiments the homogenates contained the same number of midguts per ml. The dry weight of an aliquot of the homogenate was determined after drying in an oven at 110°C. The reaction mixtures contained one half gut per ml and had a buffered azocasein concentration of ten mg per ml. After incubation at 40°C for 90 minutes enzymatic activity was stopped by precipitation with ten percent trichloroacetic acid. Reagent blanks as well as boiled enzyme blanks were utilized. Six replications were used and the activity reported in Klett units (table 1).

*Proteolytic activity of the diverticulum.*—The diverticulum or crop of the stable

fly consists of a posteriorly directed ventral diverticulum suspended by tissue from the anterior part of the midgut. In blood-fed flies it was seen as a contracted, opaque, bilobed saclike structure connected through a slender tube to the proventriculus. But, in a few cases, blood was found in the diverticulum following a blood meal. During a series of approximately 200 dissections, ten blood-filled diverticula were found and were prepared as a homogenate in pH 7.8 *Tris* buffer. In a similar manner a homogenate was prepared from ten non-blood-filled diverticula. The trypsinlike activity of both these homogenates was compared with a homogenate of midguts following an incubation of one hour at 40°C with azocasein at ten mg per ml. The concentrations of the reaction mixtures and the resulting Klett readings were as follows: 3.33 blood-filled diverticula per ml—4 Klett units; 3.33 non-blood-filled diverticula per ml—0 Klett units; and 0.5 midgut per ml—50 Klett units. These results are interpreted as being negative for both types of diverticula and, of course, positive for the midguts.

*Activity characteristic determination.*—The activity characteristic has been used to compare the activity of enzymes from various sources. Lin and Richards

TABLE 1

*Trypsinlike activity of male and female stable flies*

Test	Sex	R <sub>90</sub> *	Mg Dry Weight†
1.	Male	29	0.0172
	Female	26	0.0181
2.	Male	24	0.0183
	Female	20	0.0181
3.	Male	41	0.0150
	Female	40	0.0140
4.	Male	25	0.0216
	Female	23	0.0212
5.	Male	22	—
	Female	23	—
6.	Male	30	—
	Female	33	—

\*Activity in terms of Klett units following 90 minutes of incubation.

†Milligrams of dry weight per two midguts.

TABLE 2

*The effect of temperature on activity*

Temperature	Activity in <sup>1</sup> Klett units	Mg Substrate hydrolyzed per hour
30	18	0.126
35	30	0.211
40	45	0.316
45	67	0.470
50	69	0.484
60	40	0.281

<sup>1</sup>Average of four replications.

(1956) have used the activity characteristic to compare proteinases from the American cockroach and the house fly. In determining this parameter of stable fly midgut protease the formula substitution method was used (Neilands and Stumpf, 1955). The degree of hydrolysis of the azocasein substrate was determined by comparing the Klett readings of the enzyme substrate reaction mixture with a standard curve. Alkalinization of serial dilutions of a known amount of substrate was used to produce the standard curve.

By means of reaction mixtures containing ten mg of substrate and two-thirds of a midgut per ml, the amount of substrate hydrolysis was determined over a series of temperatures ranging from 30 to 60°C (table 2). A combined homogenate was required to avoid differences in proteolytic activity that exist between different groups of flies. Four replications were employed at each temperature. The averaged readings are reported in the table. Thermal inactivation was found to predominate above 50°C with the one hour incubation period.

*Determination of the order of reaction.*—The order of reaction was determined by plotting the activity of a homogenate azocasein reaction mixture at various

time intervals. The buffered substrate and homogenate concentrations were ten mg and one gut per ml of reaction mixture, respectively. Aliquots of the reaction mixture were removed from the incubating reaction mixture after 0, 30, 45, 60, 90, and 120 minutes. These aliquots were analyzed for hydrolysis of the substrate. Figure 1 is a plot of the substrate hydrolyzed per milliliter of reaction mixture for the different time periods of incubation. Four replications of this experiment were made.

*Effect of dialysis on activity.*—A midgut homogenate was prepared, and a known volume of it was dialyzed against distilled water overnight in the cold. Then the proteolytic activity of the dialyzed portion and of the nondialyzed homogenate was determined using one ml aliquots. Also one ml aliquots of the dialyzed and nondialyzed homogenates were analysed by Hengar Micro-Kjehldal for protein nitrogen.

Following this, the activity of the separate homogenates, dialyzed and nondialyzed, were related to units of protein nitrogen. Since the activity per mg of protein nitrogen was found to be the same in both cases no reason was found to suspect the presence of a dialyzable activator.

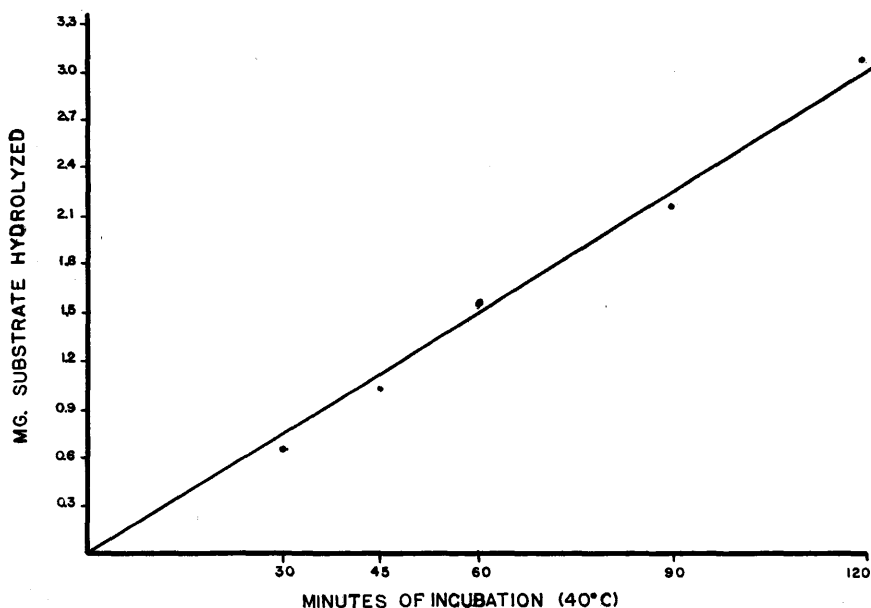


FIGURE 1. Activity recorded in terms of Klett units following incubation of 0.5 ml of homogenate with two ml of azocasein substrate after incubation at 40° C for varying periods up to 120 minutes.

*Effect of selected ions, antibiotics, and toluene on activity.*—In addition, no effect on activity was found when the dialyzed homogenate reaction mixture was made 0.02 M with respect to the following ions: calcium, magnesium, sodium, chloride, and fluoride. The effect of three antibiotics was tested on the reaction mixture. Penicillin G (Squibb) was found to have no effect on the reaction, while dihydrostreptomycin (Squibb) and terramycin (Pfizer) were found to alter the substrate, apparently resulting in nonenzymatic cleavage. When toluene was tried as a preservative the substrate tended to decompose appreciably upon overnight storage in the refrigerator. This was especially undesirable since the hydrolyzed chromophoric group of the azocasein was soluble in the toluene layer and accumulated there.

*Paper electrophoresis of the midgut homogenate.*—Paper electrophoretic techniques were adopted from procedures used in the separation of human blood plasma proteins as suggested by Dr. Robert L. Wall (1956, personal communication). The equipment has been described above.

Barbital buffer, with an ionic strength 0.05 at pH 8.0, was placed in the end cells of the electrophoresis apparatus and the tray was put between the end cells. The buffer level was adjusted with a siphon before the paper strips were arranged on the tray with their ends dipping in the end cells. A line was drawn with a pencil across the center of the paper strips. Buffer was applied to the paper by means of a medicine dropper. Then the tray was covered with a glass plate and the direct current was applied to the electrodes. After three hours of equilibrating at 1.5 mamp per strip (approximately 500 vdc), the current was disconnected and approximately 20  $\mu$ l of homogenate was placed on the pencilled line of each strip. Then the glass plate was replaced and the power, equivalent to 1.5 mamp per strip, was reapplied.

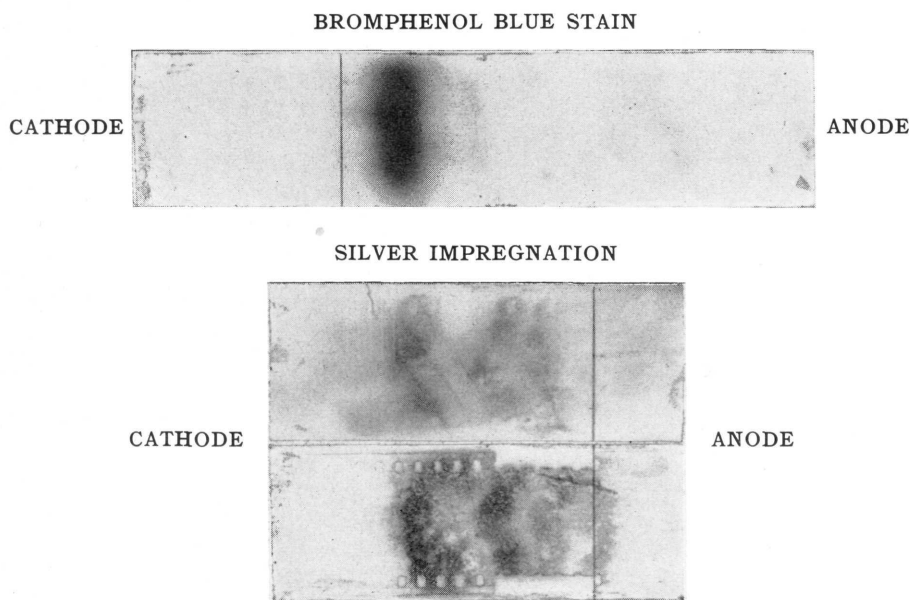


FIGURE 2. The strip stained with bromphenol blue illustrates the distribution of protein of the stable fly midgut homogenate, while the silver impregnated strips illustrate the distribution of the protease of the stable fly midgut homogenate, following paper electrophoresis.

Following electrophoresis of duplicate strips for 24 hours, one strip was used to determine the location of the protein while the other was used to test for proteolytic activity. Bromphenol blue staining identified the protein fractions (Wall, 1956, personal communication) while incubating the other strip with exposed and developed *Kodak 35 mm Plus X* film located enzyme activity. The exposed film was moistened with pH 7.8 *Tris* buffer and placed on top of the electrophoretic strip. The whole was sandwiched between two glass plates and incubated at 39°C for 45 minutes. Enzyme activity was detected by the hydrolysis of the gelatin from the film. This process released the silver particles which impregnated the paper. Figure 2 shows a bromphenol blue dyed strip and a strip stained with the free silver particles.

As indicated in figure 2, the enzymatic component exhibited cathodal movement

while the remaining protein fractions exhibited anodal migration. Although three bands of enzymatic activity could be located by use of the photographic film, either by the clearing of the film itself or by the impregnation of the silver in the paper, the enzymatic material apparently contained too little protein to stain with the bromphenol blue dye. No cathodal migration of the enzymatic material was found at either pH 7.6 or pH 8.6 with the barbital buffer.

*Starch gel electrophoresis.*—Bernfeld and Nisselbaum (1956) have used starch gel electrophoresis to separate proteins from mouse serum. This method was applied to the separation of the proteinase from the midgut homogenate of the stable fly. Bernfeld and Nisselbaum prepared a purified amylose for the starch gel. However, in the following experiments a satisfactory starch gel was formed from commercial *Cream* cornstarch as previously described. After the gel had cooled overnight in a refrigerator, a slot was cut across the gel in the center of the tray. This slot was of such a size that it could hold either one or two ml of homogenate, depending on the experiment. After filling the slot with the

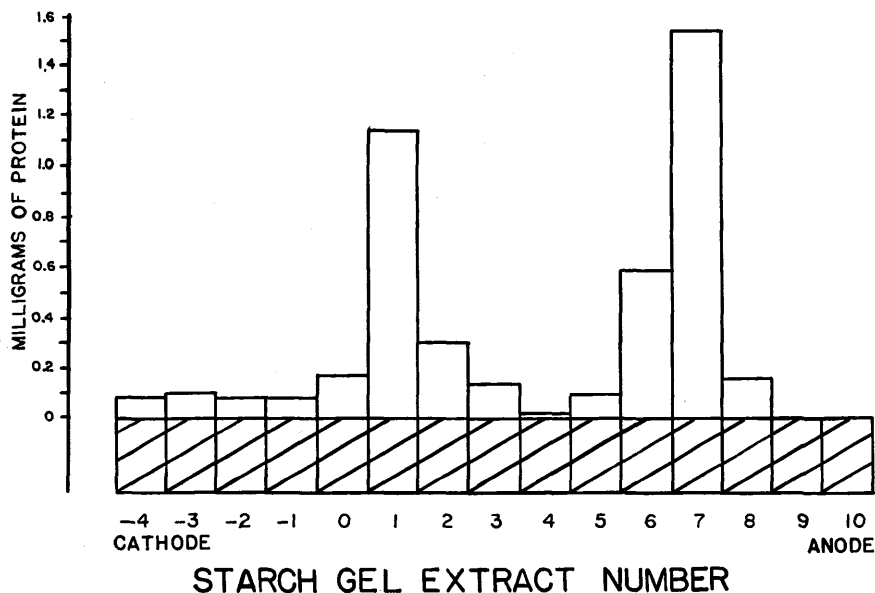


FIGURE 3. The shaded area represents the interference of the soluble starch in the Folin-Ciocalteu protein determination. The peaks of protein in the anodal sections 1 and 7 represent the location of the two proteins following starch gel electrophoresis.

homogenate, the tray was placed between the end cells of the paper electrophoresis apparatus and connected to them with filter paper wicks. The tray was covered with a glass plate and direct current applied so that each cell received 200 vdc at 12 to 15 mamp.

After 24 hours of electrophoresis, the tray was removed and the gel divided into cross sections of either one or one half cm in width. Each section of the gel was transferred to a separate centrifuge tube. Three successive extractions with 0.3 M *Tris* buffer at pH 7.8 were used to separate the protein from the gel. Each extraction consisted of adding three ml of buffer, shaking the tube manually for 30 seconds, centrifuging to throw down the starch granules, and, finally, pouring off the supernatant which contained the protein. The supernatant from each successive extraction of the individual starch sections was pooled. As a result

the protein contained in each section of the starch gel was transferred to a test tube in a measurable volume of buffer. Aliquots of the extract were used to test both enzyme activity and protein concentration.

It was found during trial runs, when four instead of three extractions were made, that successive extracts contained 53, 26, 16, and 5 percent of the total protein recovered. The Folin Ciocalteu protein determination was used to measure the amount of protein in the extract. A section of the gel which contained no protein was extracted to serve as a blank, since such soluble starch as remained in the extracts interfered in the Folin Ciocalteu test.

This procedure was capable of separating a protein mixture as illustrated by the following test. A solution composed of equal parts by weight of Armour bovine plasma fractions I (fibrinogen) and V (albumin) in 0.9 percent sodium chloride was placed on a starch gel after it had been prepared in the usual way. Following electrophoresis, at pH 8.6 in *Tris* buffer for 24 hours, the starch was

TABLE 3  
*Activity distribution following starch gel electrophoresis. Activity in Klett units per milligram of protein*

Section Number	<i>Tris</i> -Citrate Buffer			Barbital Buffer pH 8.0
	pH 7.6	pH 7.6	pH 8.0	
(Cathode)				
-3	0	0	0	0
-2	0	0	0	91
-1	410	500	500	544*
0	690*	770*	838*	260
1	410	433	640	107
2	281	321	341	188
3	171	280	303	440*
4	267	287	436*	25
5	440*	333*	412	78
6	118	311	357	244*
7	187*	293	199	90
8	136	544*	158	45
9	40	300	150	0
10	0	222	0	0
11	0	0	0	0
(Anode)				

\*Activity peak.

sectioned and extracted. When the amount of protein in each extract was measured by means of the Folin Ciocalteu procedure, 99 percent of the total protein was accounted for. It is obvious from figure 3, which illustrates the linear distribution of the protein in the gel, that the two fractions, I and V, exhibited different electromobilities.

In a further test the brei was placed in a prepared starch gel made with *Tris*-citrate buffer at pH 8.0. This brei had been previously dialyzed and lyophilized. Then after being diluted with water to a known volume, aliquots were tested for enzyme activity and protein content. On the basis of this information, the volume was adjusted so that the final protein concentration was equivalent to a two percent albumin solution.

Following electrophoresis of this homogenate, the starch gel was sectioned and extracted. Then aliquots of the extracts were tested for enzyme activity

and protein concentration. Since the volume of the extract was known, it was possible to calculate the protein and enzyme distribution in the starch gel. In addition, it was possible to determine the amount of purification by comparing the activity per mg of protein of the homogenate with the activity per mg of protein in the extracts.

Table 3 lists the relationships of activity per mg of protein of extracts from four starch gels. The degree of purification for the enzyme is given for each section of the starch gel. Inspection of this table reveals that no cathodal migration of the protein or enzyme material was found. This contrasts with the results of paper electrophoresis. The enzymatic distribution consistently appeared in several peaks but the peaks were not similarly distributed in the different gels.

One gel was made up to have the same concentration of the barbital buffer as was used in the paper electrophoresis. This gel, following extraction, exhibited no cathodal migration of enzymatic material.

TABLE 4

*Protein distribution following starch gel electrophoresis (Tris-Citrate Buffer, pH 7.6)*

Sect. No.	Milligrams of Protein Per Section		Combined Extracts		
	Gel No. 1	Gel No. 2	Sections Combined	Total mg Protein Remaining	Activity per mg Protein
(Cathode)					
-5	0.00	0.06			
-4	0.00	0.06			
-3	0.06	0.16			
-2	0.32	0.51	-1, -2	2.75	0
-1	0.52	0.84			
0	2.75	1.84	0, 1	7.50	327
1	2.92	1.89			
2	2.22	3.14			
3	1.22	2.29	2, 3, 4	2.75	227
4	0.77	1.07			
5	0.55	0.58			
6	0.17	0.56	5, 6, 7	1.50	618
7	0.41	1.11			
8	0.43	0.56			
9	0.13	0.27	8, 9	0.50	157
10	0.11	0.23			
11	0.11	0.13	10, 11	0.50	400
12	0.11	0.08			
(Anode)					

*Specific activities of the starch gel extracts.*—Enough material could be extracted from the starch gel to check the relative activity against two different substrates, sulfanilamide azocasein and sulfanilic acid azoalbumin. Two gels were prepared with pH 7.6 Tris-citrate buffer. Starch gel electrophoresis was carried out in the usual way. Following extraction and the determination of the protein distribution, those sections which apparently contained peaks of protein were combined. Table 4 lists the sections combined and the total activity per mg of protein of the combined extracts. In order to determine the activity of the extract the volume was first reduced by lyophilization. Then each extract was diluted with distilled water to a standard volume, five ml. One half ml of this was removed to test for enzyme activity. Another aliquot was removed to check the protein content. Then by accurately adjusting the volume of each combined extract, it was possible to



produce extracts having approximately the same amount of activity per unit of volume. Following this the activity of each extract was measured with the two substrates listed above. The results are given in table 5. A comparison in the form of a ratio of the activities with the two substrates, is presented in the last column. Presumably, if the separate extracts contained the same enzyme, the ratios would be identical. Since this was not found (table 5), this study indicated that the homogenate of the stable fly midgut contained a variety of different proteolytic enzymes of the trypsinlike type or least two (or more) enzymes in a variety of proportions.

## DISCUSSION

Data in table 1 indicate that no differences exist in trypsinlike activity of midgut homogenates of either sex, providing that the homogenates are from the same group of flies. The term group refers to a population of flies reared from one aggregation of pupae which was subsequently handled in the same manner. Different groups of flies could not be compared in terms of activity per midgut or in terms of activity per unit of dry weight. Because of this, enough homogenate was prepared to supply the requirements for any experimental procedure prior to the actual experimentation.

TABLE 5  
*Specific activity of combined extracts*

Extract Number	Sections Combined (Table 4)	Activity <sup>1</sup>		Activity Ratio
		Azocasein	/ Azoalbumin	
(Cathode)				
1	0, 1	36.5	/ 8	= 4.6
2	2, 3, 4	48	/ 23	= 2.1
3	5, 6, 7	48	/ 14	= 3.4
4	8, 9, 10, 11	40.5	/ 16	= 4.6
(Anode)				

<sup>1</sup>Activity is an average of two determinations and is reported in terms of Klett units.

The activity characteristic was found by the formula method to be 15,600 calories. The formula substitution method of Neilands and Stumpf (1955) using the equation

$$k_2/k_1 = \frac{0.219 \mu (T_2 - T_1)}{T_1 T_2} \quad \text{was used.}$$

$K_1$  and  $k_2$  represent the activity at absolute temperatures  $T_1$  and  $T_2$ . The temperature range selected was from 30 to 50°C. Lin and Richards (1956) have reported characteristics of 15,000 to 16,000 calories for the American cockroach using similar procedures. Hog trypsin was found to have a temperature characteristic of 15,600 calories with a casein substrate (Sizer, 1942).

Charney and Tomarelli (1947) report that vertebrate trypsin catalyzes the hydrolysis of azocasein and that this reaction follows the first order type. However, the curve in figure 1 demonstrates a zero order reaction since the substrate hydrolysis is directly related to time. Charney and Tomarelli used a higher substrate concentration, 20 mg per ml of reaction mixture, than was used in the stable fly midgut reaction mixture. Perhaps if the midgut concentration had

been increased or the substrate concentration decreased, a first order reaction might have resulted with the stable fly midgut protease.

The midgut homogenate was found to retain the same proteolytic activity in terms of protein nitrogen following dialysis against distilled water as it possessed prior to dialysis. This demonstrated that a dialyzable activator was not present. The addition of the cations, sodium, calcium, and magnesium, or the anions, chloride and fluoride, did not alter the rate of activity of the dialyzed homogenate.

Penicillin G was found to have no effect on the midgut trypsinlike activity. This indicated either that bacterial contamination was not present or it was unaffected by the antibiotic. Terramycin and dihydrostreptomycin were found to alter the substrate which in turn resulted in abnormally high activities with the midgut preparation. Nor was toluene a satisfactory substrate preservative since its use resulted in nonenzymatic cleavage and the hydrolyzed chromophoric group was soluble in the toluene layer. It was found best to keep the substrate solution frozen. This inhibited the hydrolysis of the azocasein.

Some confusion has been introduced into the literature by the statement of Champlain and Fisk (1956) that: "In the muscoid bloodsuckers, such as *Glossina* and *Stomoxys*, ingested food passes into the crop, where no digestion or denaturation occurs." It is now generally agreed that a more accurate appraisal is given in the well documented statement of Waterhouse (1957), namely: "Tabanids, *Phlebotomus*, and the stable fly *Stomoxys* also divert blood to the midgut and sugar solutions to the crop, whereas *Glossina* sends blood to both regions."

During laboratory feeding of stable flies with citrated bovine blood, blood entered the ventral diverticulum or crop in less than five percent of the flies. When these blood-filled diverticula were incubated with azocasein substrate at pH 7.8 no proteolytic activity was found. Nor was proteolytic activity detected in diverticula which showed an absence of ingested blood. Flies reared on sugar solutions were found to have enlarged clear fluid-filled diverticula indicating that the carbohydrate solutions had been taken into these structures. The midguts of these flies were smaller than in the blood-filled flies. Because of this, it seems likely that most of the carbohydrate material was taken into the diverticulum instead of the midgut.

At least three separate bands of trypsinlike activity were found following paper electrophoresis of the stable fly midgut homogenate. Paper electrophoresis was carried out with barbital buffer at pH 8.0. Since the amount of brei—20 microliters—was too small to elute from the paper, further characterization of this enzyme by this method was not attempted. Starch gel electrophoresis yielded enough enzymatic protein to test against two separate protein substrates, sulfanilamide azocasein and sulfanilic acid azoalbumin. In contrast to paper electrophoresis no cathodal migration of the enzymatic material was found after starch gel electrophoresis. This was true even though the buffer composition used in making up the starch gel and the pH of electrophoresis was the same as in paper electrophoresis. The reason for the difference in migratory behavior remains unknown. Perhaps it is associated with the nature of the electrophoretic substrate.

Another difference between the two methods was that the homogenate used with starch gel electrophoresis had been lyophilized and dialyzed while the homogenate used with paper electrophoresis had been untreated. However, the dialyzing and lyophilizing treatments have been shown to have no effect on total protease activity.

The distributions of the nonenzymatic protein and of the enzymatic material following starch gel electrophoresis was found to be independent of each other. This is illustrated in table 3 as the enzymatic content of the extracts did not vary proportionally to the nonenzymatic protein component of the serial extracts.

By comparing the activity per mg of protein of the original homogenate with

the activity per mg of protein in the various extracts, the degree of purification achieved following electrophoresis was determined. The highest degree of purification was found in the combined extracts of section four as listed in table 4. This amounted to a purification of 14-fold.

Some preliminary work was done on the possibility of using acetone and salt fractionation at different pH levels and temperatures to purify the enzyme. The highest purification obtained with a one-step acetone fractionation was about 14-fold. However, the total recovery of enzyme was only 28 percent. This purification was obtained in the supernatant of a homogenate buffer preparation which contained 20 midguts per three ml after it had been treated for four hours with an equal volume of acetone at 12° C. Then it was found that by shifting the pH to 9.0 (at the same temperature) the enzyme material could be precipitated with an additional two-fold purification. In this step there was an additional 72 percent loss in activity. Since this procedure required large amounts of homogenate, purification by this method was discontinued.

As previously mentioned, enough material was obtained in the starch gel extracts to study the comparative activities of the different extracts with the two available substrates. If the volumes of the extracts could be adjusted to the same enzymatic activity per unit of volume, using one substrate as a standard, then each extract should have the same relative activity when tested against a second substrate if the same enzyme was present in each. If the extracts contained qualitatively different proteolytic enzymes, then the activities of the extracts in the second substrate would be expected to vary. However, it was found difficult to adjust the volumes of the extracts accurately so that the different extracts would have the same activity per unit of volume with one substrate. Instead, the volumes of each were adjusted so that they had as nearly as possible the same concentration of the enzyme. A pipette graduated in hundredths of a milliliter was used to adjust the volumes of the extracts following the first activity determination.

Then, in duplicate experiments, the same volume of each extract was added to each of two test tubes. The same clean dry 0.5 ml pipette was used for each transfer. After equilibration in the 40° C water bath, a two ml aliquot of one substrate was added to one of the tubes representing each extract. The second substrate was added to the other test tube. Appropriate water blanks were tested simultaneously. This entire procedure was repeated and the results averaged.

At the end of the incubation period the reaction was stopped and the activity of each extract with the two substrates was compared. This was done in the form of a ratio as listed in table 5. The ratio for each extract should be the same if the enzyme contained in the extracts was of the same type. But the activity ratio was found to vary for the different extracts. This is a good indication that some qualitative difference existed in the proteolytic enzyme of the extracts.

This evidence, combined with the appearance of three activity peaks found in paper electrophoresis, leads to the conclusion that the midgut trypsinlike enzyme of the stable fly is a complex of trypsinlike enzymes. Perhaps this has some bearing on the different enzymatic activities of homogenates prepared from different groups of flies. That is, variation in the physiological condition of the flies and of the bovine blood might affect the constitution of the trypsinlike enzymes secreted into the gut.

#### SUMMARY

In a study of the midgut trypsinlike enzymes of the stable fly, *Stomoxys calcitrans* (L.), no trypsinlike activity was found in the diverticulum. The diverticulum was found to contain blood in less than five percent of the flies following laboratory feeding of citrated bovine blood. Also no difference was

noted in the trypsinlike activities of midgut homogenates prepared from male and female flies taken from the same population of flies.

Differences were found in homogenates prepared from different groups of flies. The trypsinlike proteolytic action was found to follow the zero order type of reaction at the concentrations of substrate and homogenate used. No evidence of a dialyzable activator was found. The addition of certain metallic ions—magnesium, sodium, and calcium—and the anions—chloride and fluoride—to dialyzed homogenates did not affect the reaction.

Paper electrophoresis of the homogenate indicated that the midgut contained at least three trypsinlike substances which possessed different electromobilities. Two of these bands moved toward the cathode while the third remained stationary at pH 8.0 using barbital buffer of ionic strength 0.05. An attempt was made to increase the yield by electrophoresis of larger quantities of homogenate by the use of starch gel electrophoresis. However, no cathodal movement of the enzyme was found in the starch gel. Serial extractions of the starch gels demonstrated different peaks of activity on the anodal side of the starting point. A comparison of the activity of several of the peaks with different substrates demonstrated the likelihood that the peaks represented different trypsinlike components of the stable fly midgut protease.

#### ACKNOWLEDGMENTS

This study was supported in part by Research Grant E-485 (C4) from the National Institutes of Health, U. S. Public Health Service. We wish to thank Dr. R. A. Champlain and Dr. E. N. Lambremont for the preparation of the diazotized azocasein and azoalbumin used in this investigation.

#### LITERATURE CITED

- Bernfeld, P. and J. S. Nisselbaum.** 1956. A modified method for protein separation by zone electrophoresis on a starch gel. *Jour. Biol. Chem.* 220: 851-860.
- Champlain, R. A.** 1955. The digestive enzymes of the stable fly, *Stomoxys calcitrans* (L.). Ph.D. Dissertation, The Ohio State University. 83 pp.
- and **F. W. Fisk.** 1956. The digestive enzymes of the stable fly, *Stomoxys calcitrans* (L.). *Ohio Jour. Sci.* 56: 52-62.
- , **F. W. Fisk,** and **A. C. Dowdy.** 1954. Some improvements in rearing stable flies. *Jour. Econ. Ent.* 47: 940-941.
- Charney, J. and R. M. Tomarelli.** 1947. A colorimetric method for the determination of proteolytic activity in duodenal juice. *Jour. Biol. Chem.* 171: 501-505.
- Lin, S. and A. G. Richards.** 1956. A comparison of digestive enzymes in the house fly and American cockroach. *Ann. Ent. Soc. Am.* 49: 239-241.
- Lowry, O. H., N. J. Rosenbaum, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin-Phenol Reagent. *Jour. Biol. Chem.* 193: 265-268.
- Neilands, J. B. and P. K. Stumpf.** 1955. *Outlines of Enzyme Chemistry.* John Wiley & Sons, Inc. New York. 315 pp.
- 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.* 34: 428-433.
- Waterhouse, D. F.** 1957. Digestion in insects. *Ann. Rev. Ent.* 2: 1-18.