STUDIES ON THE DIGESTION OF WOOL BY INSECTS

II. THE PROPERTIES OF SOME INSECT PROTEINASES

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Summary

The effects of a number of protease activators and inhibitors, namely cyanide, iodoacetate, thioglycollate, enterokinase, ovomucoid, *Ascaris* inhibitor, and soybean inhibitor, have been studied on crude proteinase preparations from the midguts of the following five species of insects: *Tineola bisselliella* larvae, *Tenebrio molitor* larvae, *Musca domestica* larvae, *Periplaneta americana*, and *Locusta migratoria*, and the results compared with their effects on trypsin and papain. Similarly, a comparison has been made of the pH optima, heat stability, and milk-clotting ability of these enzyme preparations and of the effects of oxidation-reduction potential on their hydrolytic ability. In general, the insect enzymes are similar, and resemble trypsin more closely than papain. Those differences that are found between enzymes from the sources mentioned indicate that the special ability of the larva of the clothes moth *Tineola* to digest keratin does not reside in its protein-digesting enzymes.

I. Introduction

It is generally considered that "the digestive enzymes of insects resemble those of other animals; they are activated and inhibited by the same reagents, they are similarly affected by changes in pH and so forth" (Wigglesworth 1939). However, Linderstrom-Lang and Duspiva (1936) showed that the proteinase of *Tineola* larvae differed from vertebrate pancreatic trypsin in that it was not inhibited by sulphydryl-containing compounds, and they considered this to be a useful adaptation to the substrate because of the high concentration of cystine in the diet of clothes moths. Duspiva (1936) found that the proteinase of the larva of the wax moth *Galleria* reacted to thioglycollic acid in a manner intermediate between *Tineola* proteinase and trypsin. The question has remained unanswered whether these lepidopterous larvae differ from other insects in their comparative insensitivity to sulphydryl compounds, or whether all insect proteinases differ from trypsin in this respect.

In order to answer this question, and to determine whether the digestive proteinase of *Tineola* differs from those of other insects in other features, which might account for its peculiar ability to digest keratin, the experiments reported below were undertaken. It was found that the differences between insect proteinases, although demonstrable, were not marked. However, a number of exceptions to Wigglesworth's generalization, quoted above, became apparent.

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II. Methods

(*a*) *Proteinase Preparations*

Proteinase preparations were obtained from adults of *Periplaneta americana* (L.) and of *Locusta migratoria* (L.); larvae of *Tenebrio molitor* L., larvae of *Musca domestica* (L.), and larvae of *Tineola bisselliella* Humm. These were prepared for the experiments with activators and inhibitors by homogenizing the freshly dissected midguts and contents in 0.067M phosphate buffer at pH 8.0. Only caeca were used with *Periplaneta* and *Locusta*. These preparations were compared with trypsin (B.D.H.) and papain (commercial preparation). Details of a typical series of these preparations are given in Table 1.

Table 1

DETAILS OF ENZYME PREPARATIONS FOR INHIBITOR EXPERIMENTS

Source of Enzyme	Number of Insects/ml.	Proteinase Activity (optical density)	Protein N (mg./ml.)
Periplaneta	0.6	0.271	0.048
Locusta	0.024	0.264	0.089*
Tenebrio	4.25	0.238	0.848
Musca	7.5	0.263	0.248
Tineola	47	0.233	0.272
Trypsin	0.310†	0.270	0.033*
Papain	7.45†	0.248	0.252

* Total nitrogen.

† Mg./ml.

For the examination of the effect of heat on the enzymes, a phosphate-borate buffer at pH 6.0 was used for papain, a carbonate-borate buffer at pH 10.0 for *Tineola*, and phosphate-borate buffer at pH 8.0 for the other enzymes. For all other experiments, water extracts of acetone-dried material were used.

(*b*) *Activators and Inhibitors*

(i) *Proteinase Determinations.*—These were performed by the method of Charney and Tomarelli as described by Day and Powning (1949), except that half the quantity of double concentration of azocasein substrate was used to permit addition of reactants.

(ii) *Activators and Inhibitors of Proteases.*—There were prepared as follows:

(1) Sodium cyanide—0.5M solution adjusted to pH 8.0 with HCl.

(2) Thioglycollic acid—neutralized by slow addition of NaOH while cooling in an ice bath. The thioglycollic acid was added as a 0.45M solution, but, owing to the tendency to rapid oxidation, the concentration was reduced. Its effect on papain, however, indicated that it was an active preparation.

(3) Sodium iodoacetate—0.5M solution adjusted to pH 8.0 with NaOH.

(4) Ovomucoid—prepared according to the trichloracetic acid-acetone precipitation method of Lineweaver and Murray (1947).

(5) Enterokinase—prepared by water extraction from recently acetone-ether-dried pig intestinal mucosa (Hawk, Oser, and Summerson 1947, p. 360).

(6) *Ascaris* inhibitor—prepared according to the shorter method of Collier (1941).

(7) Soybean inhibitor—prepared by extraction at pH 4.2 and purified with kaolin and acetone (Ham and Sandstedt 1944).

(*c*) *pH Optima*

An attempt was made to use the azocasein method for this work, but it was discarded in favour of the gelatin viscosity method of Lennox (1943). The enzyme concentrations were adjusted to give about 15-20 per cent. reduction in viscosity. Five ml. of 10 per cent. gelatin at the required pH was mixed with 1.8 ml. water in a viscometer and kept in a water bath at 35.5°C. Viscosity readings (outflow times) were taken, 0.2 ml. enzyme was added and after a digestion period of 15 minutes a further viscosity reading was taken. The amount of digestion at the various hydrogen ion concentrations was plotted as percentage reduction in viscosity. A correction was made on each figure for the dilution by the enzymes.

(*d*) *Milk Clotting*

Milk clotting was studied by the method of Balls and Hoover (1937). All enzymes were adjusted to the same concentration by estimation with the azocasein proteinase method at pH 8.0.

(*e*) *Heat Stability*

Proteinase determinations were carried out using the normal concentration azocasein substrate adjusted to the same pH as the enzyme solutions. Aliquots of 0.5 ml. of enzyme solution were heated in tubes in a water bath for various times at temperatures varying from 60°C. to boiling, then, on removal and cooling, 0.5 ml. of substrate was added and the tubes incubated at 37°C. for 1 hr. Curves were drawn relating residual activity to time and temperature of heating and the times for the destruction of one-half of the activity reported.

(*f*) *Oxidation-Reduction Potential*

(i) *Consideration of Methods.*—Considerable difficulty was experienced in developing a satisfactory method for the examination of the effects of oxidation-reduction potential on proteinase activity. The problem consisted, firstly, of establishing and measuring a range of stable oxidation-reduction potentials in the digestion mixture, and secondly, of estimating the amount of digestion. An attempt was made to use the electrolytic technique of Hanke and Katz (1943). The oxidation-reduction potential was maintained at various levels under nitrogen by direct current electrolysis using a platinum anode or cathode, depending upon whether a high or low potential was required. The method was found to be impracticable under our conditions owing to the very poor poising in the

solutions and the rapid changes in pH that occurred with electrolysis. A technique using partially reduced dyes as poising agents and a formol titration for digestion failed because of the difficulty of keeping the oxidation-reduction potential constant for periods of time longer than one hour.

The technique that yielded constant oxidation-reduction potentials and a minimum reaction time was one in which $Na_2S_2O_4$ was added to a gelatin substrate to adjust the oxidation-reduction potential to a value at which an appropriate oxidation-reduction indicator in the solution was partially reduced, and reduction in gelatin viscosity taken as measure of digestion.

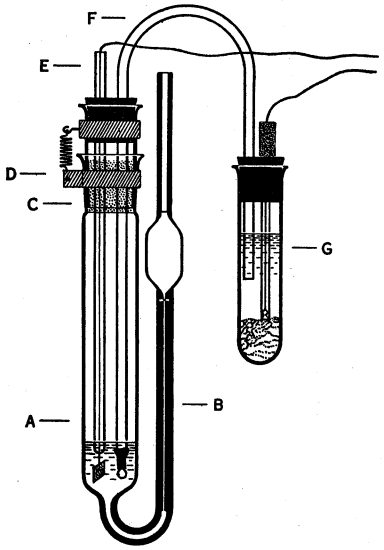

Fig. 1.—Diagram of reaction vessel for determining effect of oxidation-reduction potential on proteolysis. *A*, reaction vessel; *B*, viscometer; *C*, ground-glass joint; *D*, spring clip; *E*, platinum electrode; *F*, KCl bridge; *G*, calomel half cell. Mixing of the contents of vessel *A* was accomplished by bubbling air or nitrogen, depending on whether high or low oxidation-reduction potentials were required.

The reaction vessel (Fig. 1) was a Pyrex tube 20 cm. long and 2.5 cm. diameter. The bottom of the vessel was sealed to a viscometer side-arm of about 3 ml. capacity and having an outflow time of about 25-30 seconds for water at 35.5°C. The vessel was attached by a ground-glass joint to an adjustable stand, which could be lowered into an oil bath at 35.5°C. A platinum electrode and salt bridge from a calomel electrode were permanently mounted in the stand for oxidation-reduction potential measurements. The procedure was as follows: 5 ml. of diluted gelatin at pH 8.0 and 0.5 ml.

of dye solution were pipetted into the reaction vessel and warmed to 35.5°. Readings of outflow time were taken to check the constancy of the gelatin viscosity. Sufficient freshly prepared 0.1M $Na_2S_2O_4$ solution was added to convert half the dye to the reduced form, and the total volume of the mixture was made up to 5.8 ml. with water. Then 0.2 ml. of enzyme solution was added and the mixture was stirred with purified nitrogen.

Viscosity readings were taken after 5 and 15 minutes digestion. Proteinase activity was measured as percentage reduction of gelatin viscosity after 15 minutes. A correction was made to all results to account for the dilution effect of adding the $Na_2S_2O_4$ and enzyme by subtracting the reduction in viscosity resulting from the addition of an equivalent quantity of water. A control test with water only was carried out for each enzyme. The data are reported as differences between activity of the test mixtures and of controls containing no poising system.

The gelatin substrate was purified and prepared as a 12 per cent. stock solution according to Waksman and Davison (1926). This was kept in small quantities preserved with thymol in the refrigerator. When required, the stock solution was diluted to 3 per cent. with 0.067M phosphate buffer at pH 8.0 and kept overnight at 35.5°C. to stabilize the viscosity.

Oxidation-reduction potential readings were taken with a Cambridge potentiometer at the same time as the viscosity measurements. They were generally constant within ± 1 mv. throughout the digestion period; in a few tests, however, they varied up to ± 20 mv.

(ii) *Poising Systems Used.*—The following dyes were chosen:

Dyes	Eo (mv.)
Disodium 2,6-dibromobenzenoneindo-3-carboxyphenol	+ 250
1-Naphthol-2-sodium sulphonate-indophenol	+ 123
Methylene blue	+ 11
Potassium indigodisulphonate	− 120
Rosinduline	− 281
Benzyl viologen	− 359

(iii) *Adjustment of Oxidation-Reduction Potential of Gelatin with Other Reactants.*—Further experiments were carried out using other inorganic oxidizing and reducing agents to give a wide range of oxidation-reduction potentials. The reactants used were 0.1M $Na_2S_2O_4$, 0.1M $K_2S_2O_8$, 0.1M $K_3Fe(CN)_6$. The technique was essentially the same as described for the dyes; 5 ml. of gelatin and 0.8 ml. of reactant were mixed, the initial pH and viscosity checked, and then 0.2 ml. of enzyme was added. Control tests were carried out on each enzyme by replacing the oxidizing or reducing agents by water. The enzyme activity was measured as percentage reduction in gelatin viscosity after a 15-minute digestion period.

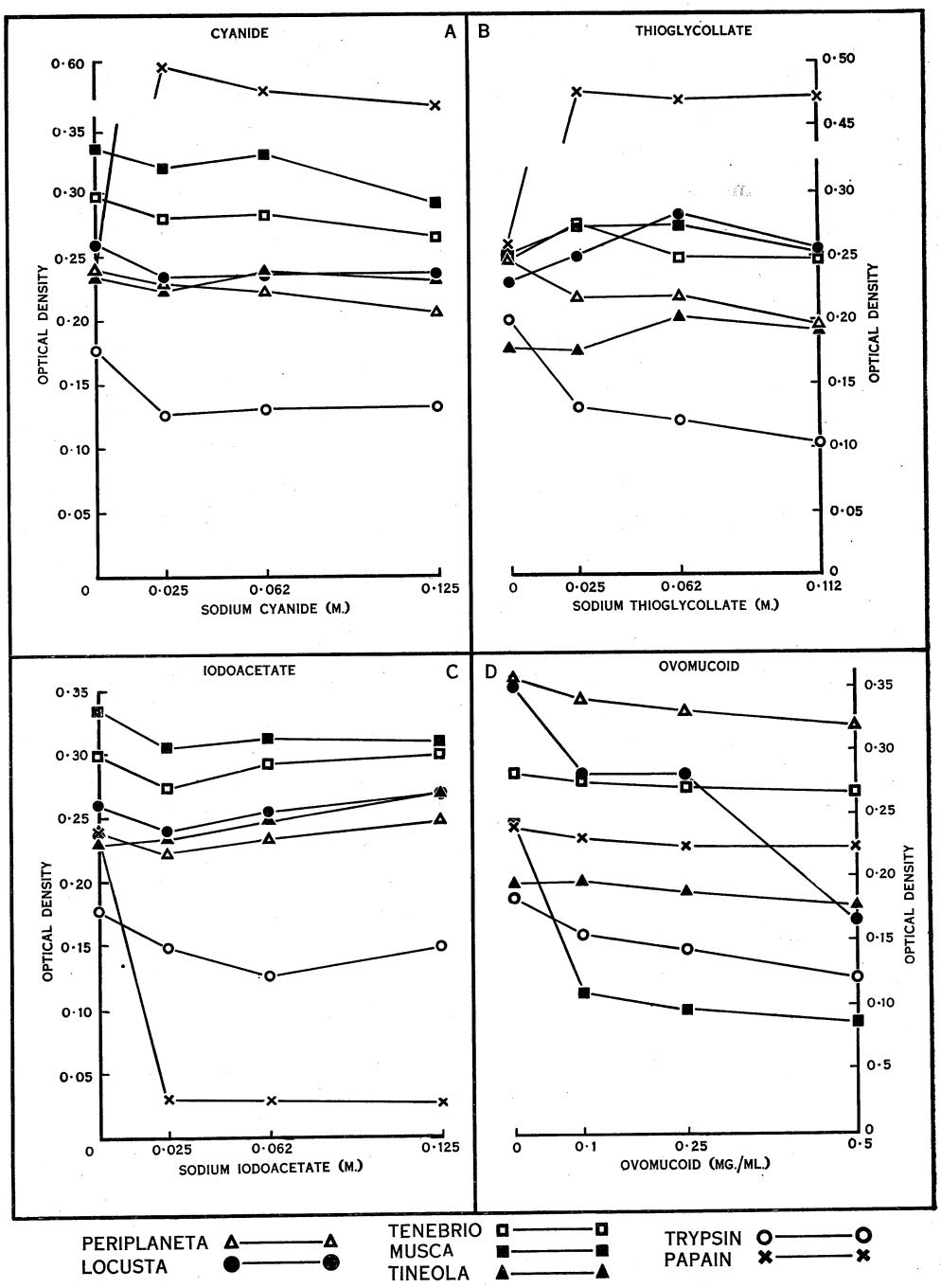

Fig. 2.—Effects of activators and inhibitors on proteinases.

III. Observations

(*a*) *Effects of Activators and Inhibitors on Proteinases*

The results are presented in Figures 2*A-D* and 3*A* and *B*. The concentrations used were those effective on trypsin and papain.

(i) *Cyanide* (Fig. 2*A*).—Papain was activated markedly, trypsin was slightly inhibited, but all the insect enzymes were unaffected.

(ii) *Thioglycollate* (Fig. 2*B*).—It is clear from the results that, at the concentrations employed, thioglycollate activated papain strongly, and inhibited trypsin. It had comparatively little effect on the insect enzymes.

(iii) *Iodoacetate* (Fig. 2*C*).—Iodoacetate strongly inhibited papain, but trypsin and the insect enzymes were not markedly affected.

(iv) *Ovomucoid* (Fig. 2*D*).—As was expected, trypsin was strongly inhibited. However, there are certain surprising differences in the reactions of the different insect enzymes. Those from *Locusta* and *Musca*, for example, were both inhibited, but those from *Tineola, Periplaneta, Tenebrio,* and papain were unaffected.

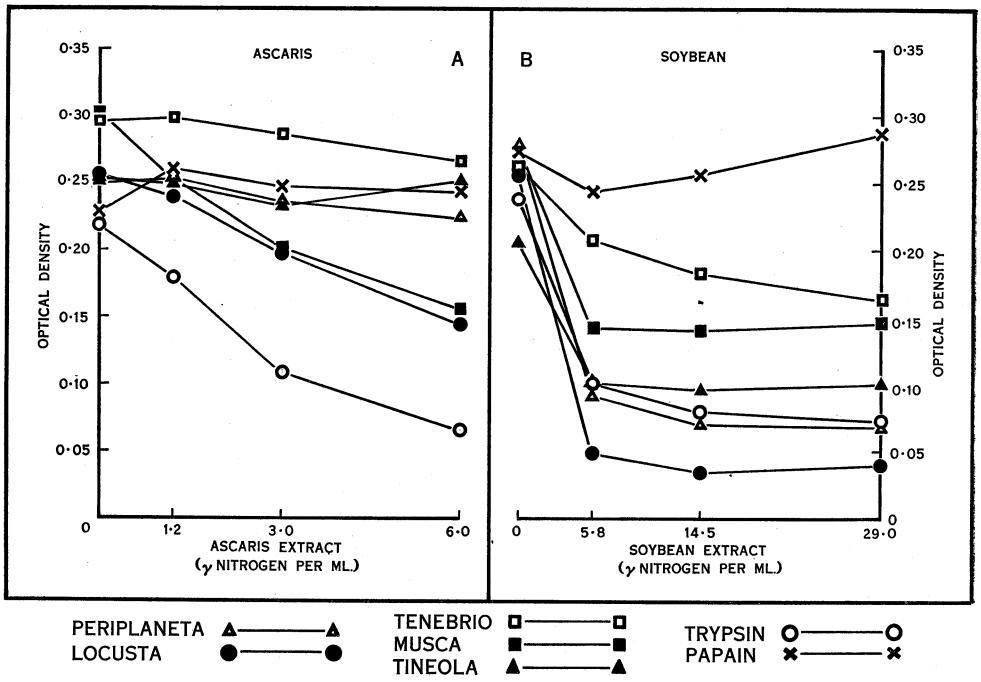

Fig. 3.—Effects of inhibitors on proteinases.

(v) *Enterokinase.*—The effect of enterokinase on trypsin was only slight, but this was due to the fact that the trypsin used was almost fully activated. However, a confirmatory test using the same enterokinase and trypsin prepared

from freshly acetone-ether-dried pancreas showed that the enterokinase was highly active; it caused an increase in activity of this trypsin from 0.099 to 0.550 units of optical density. Enterokinase had no effect on papain or on any of the insect enzymes tested.

(vi) Ascaris *Inhibitor* (Fig. 3A).—This inhibitor gave similar results to those of ovomucoid—trypsin, *Musca,* and *Locusta* were inhibited, while the other enzymes were not.

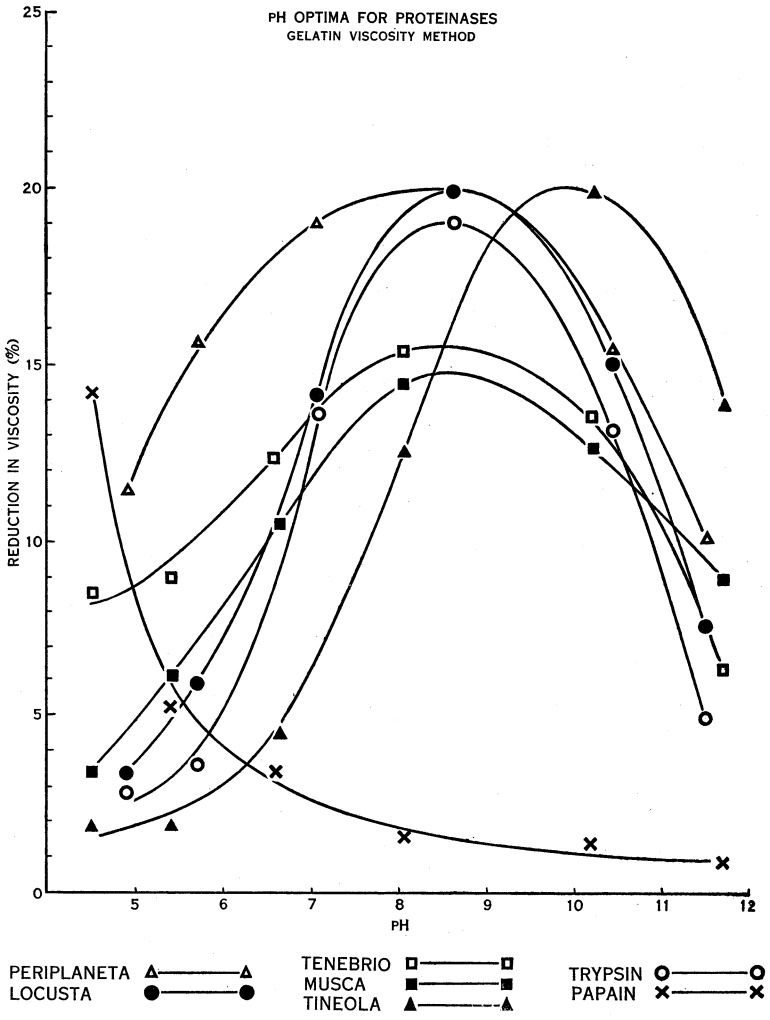

Fig. 4.—Effects of pH on proteinases.

(vii) *Soybean Inhibitor* (Fig. 3*B*).—Papain was unaffected by the soybean extract at the concentration used, but all the other enzymes were inhibited, to varying degrees. The enzyme from *Tenebrio* was inhibited least and that from *Locusta* to the greatest extent.

(*b*) *Effect of pH on Proteinases*

The curves in Figure 4 show that, with the gelatin method under the conditions used, trypsin had an optimum pH of about 8.5 and, as expected, papain had its greatest activity at a pH lower than 6. Measurements were not made below pH 4.5. Of the insect enzymes, that from *Tineola* is outstanding as its optimum is about pH 9.8-10, and its activity rapidly falls off below this. All the other insect proteinases have the same pH optimum as trypsin. However, the enzymes from *Periplaneta, Musca,* and *Tenebrio* have a wider range of activity than those from the other insects examined.

TABLE 2
MILK-CLOTTING ACTIVITY OF PROTEASES

Source of Enzyme	Action on Azocasein at pH 8.0 (optical density)	Action on Milk at pH 4.6 (mean clotting time, min. and sec.)
Periplaneta	0.25	5-44
Locusta	0.29	7-25
Tenebrio	0.26	4-52
Musca	0.29	15-5
Tineola	0.29	103
Papain	0.27	6-42
Trypsin	0.29	52

(*c*) *Milk-Clotting Activity of Proteases*

When standardized with the azocasein method at pH 8.0 to the same proteinase activity, the ability of the various enzymes to clot milk at pH 4.6 differed (Table 2). The enzymes had the same order of activity, with the exception of the enzyme from *Tineola* and of trypsin, which clotted milk very slowly. This apparently anomalous behaviour of the *Tineola* proteases undoubtedly can be explained by the differences in pH optimum (cf. Fig. 4).

TABLE 3
TIME IN MINUTES FOR DESTRUCTION OF HALF ENZYME ACTIVITY AT VARYING TEMPERATURES

Source of Enzyme	pH of Extract	Temperature				
		98°C.	90°C.	80°C.	70°C.	60°C.
Periplaneta	8.0	<1	<1	<1	1½	23
Locusta	8.0	<1	<1	<1	<1	13
Tenebrio	8.0	<1	<1	<1	2	4
Musca	8.0	<1	<1	<1	<1	15
Tineola	10.0	<1	<1	<1	<1	1
Trypsin	8.0	<1	<1	<1	1½	3
Papain	6.0	<1	1¾	13	120	510

(*d*) *Heat Stability of Proteinases*

Although the enzyme solutions were crude extracts, and conclusions based on small differences were unwarranted, it is clear from Table 3 that papain is much more resistant to thermal inactivation than any of the other enzymes.

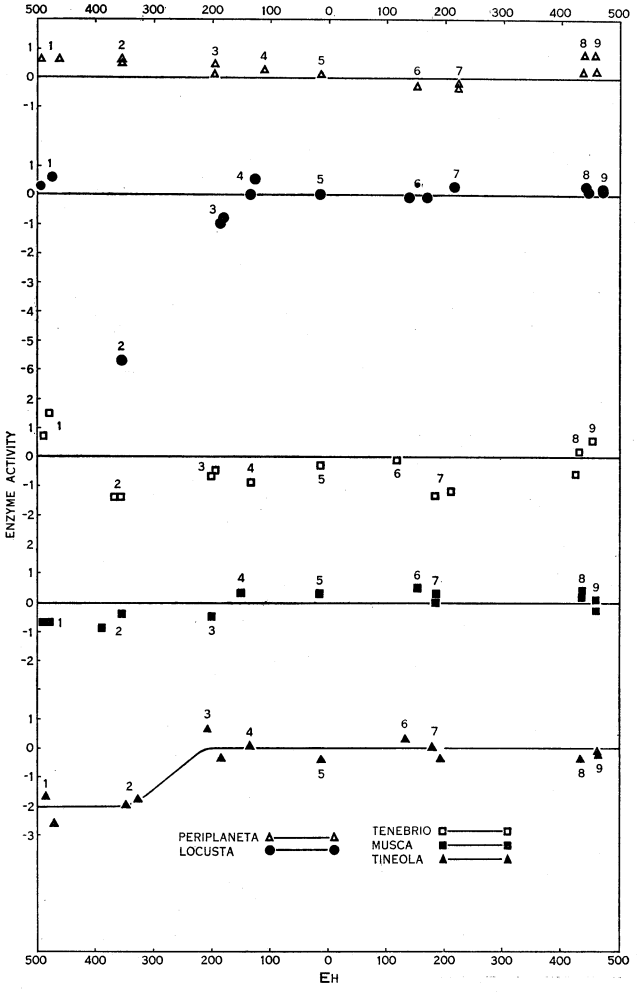

Fig. 5.—Effects of oxidation-reduction potential on proteinases. Numbers on the graphs refer to poising systems, which are as follows: 1, sodium hydrosulphite; 2, benzyl viologen; 3, rosinduline; 4, potassium indigodisulphonate; 5, methylene blue; 6, 1-naphthol-2-sodium sulphonate-indophenol; 7, disodium 2, 6-dibromobenzenoneindo-3-carboxyphenol. 8, potassium persulphate; 9, potassium ferricyanide.

Enzyme activity in the absence of a poising system is taken as zero. Differences between means are significant at the 1 per cent. level when they exceed 1.6 units, and at the 5 per cent. level when they exceed 1.2 units.

Tineola proteinase is similar to trypsin and to the other insect enzymes, except that from *Periplaneta*. This enzyme appears to be slightly more resistant to heat.

(*e*) *Effects of Oxidation-Reduction Potential on Proteinases*

The data from a large number of determinations of the effect of oxidation-reduction potential on proteolytic activity are summarized in Figures 5 and 6. The results are reported as enzyme activity in the treated samples relative to the activity of the water controls. It will be observed that the Eh varied from about − 460 to + 460 mv. Although the physiological range was covered, only relatively slight changes in activity of the insect proteinases and of trypsin were demonstrated by the methods employed.

The apparent effect of methylene blue on papain was found to be the result of poisoning. This was demonstrated by the effect of different concentrations at the same potential (Table 4).

TABLE 4

EFFECTS OF INCREASED CONCENTRATIONS OF METHYLENE BLUE ON PAPAIN ACTIVITY

Final Concentration of Methylene Blue (%)	Eh (mv.)	Activity (%)
0.0083	− 14	54.6
	− 10	49.4
0.00083	− 12	64.9
	− 4	64.5

Benzyl viologen probably poisoned *Locusta* proteinase, as indicated by the fact that $Na_2S_2O_4$, giving a lower potential, did not reduce the activity of this enzyme.

IV. DISCUSSION

In the work described above it would have been preferable to use purified enzyme preparations. This, however, was not practicable because of the large quantity of material required for purification. It was considered that sufficient indication of the enzyme properties could be obtained from crude extracts. The results with these extracts will be considered in relation to work on enzymes from other sources.

(*a*) *Effects of Activators and Inhibitors*

The failure of cyanide and thioglycollate to activate insect proteinases, and of iodoacetate to inhibit them, differentiates these enzymes sharply from many plant proteases. The absence of inhibition by cyanide and thioglycollate, and of activation by enterokinase, also differentiates them from trypsin of vertebrate origin. Schlottke (1937*b*) has claimed a small degree of activation by enterokinase of proteinase from *Carabus* and *Periplaneta*. On the other hand, the observation that trypsin is 25 times more sensitive to ovomucoid than any other proteinase studied by Fraenkel-Conrat, Bean, and Lineweaver (1949) suggests that those proteinases from insects that are similarly sensitive are closely related to trypsin in this respect.

Like the plant proteinases, those from insects have not been found capable of further activation and we cannot account for the discrepancy between our results and those reported for *Periplaneta* by Schlottke (1937*b*) with respect to enterokinase.

The inhibition of the proteinases from *Musca* and *Locusta* by ovomucoid and the *Ascaris* inhibitor suggests a similarity in mode of action of these two substances, and indicates that some differences exist between the enzymes from different insects.

Similarly, the inhibition of *Carabus* and *Tettigonia* proteinases by cyanide and cysteine (Schlottke 1937*a*) shows that the enzymes from these species differ from those of the species studied in the present paper. Additional differences between insect proteinases are suggested by the other factors studied.

(*b*) *pH Optima*

The pH optima of the insect proteinases examined vary somewhat from figures previously published (Table 5). All the values reported from the literature were determined by formaldehyde titration, except that for *Tineola* larvae, for which the Willstätter alcohol titration was used.

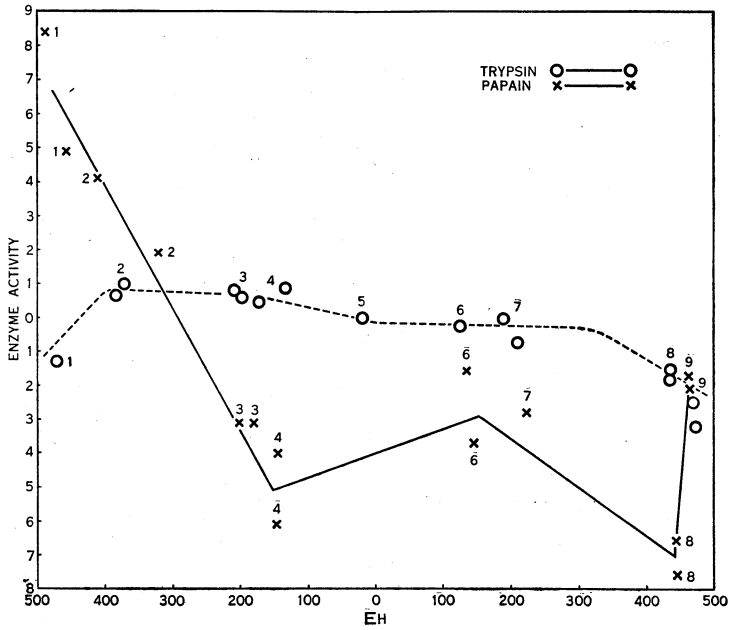

Fig. 6.—Effects of oxidation-reduction potential on proteinases. Details as in Figure 5.

These differences may all probably be accounted for by the differences in methods employed. However, under standardized conditions, the pH optima of the insect proteinases studied do demonstrate a difference between the enzyme from *Tineola* and other insects; this difference cannot be due to the impurity

of the extracts. It is correlated with considerable alkalinity of the midgut contents, which is characteristic of the Lepidoptera (Waterhouse 1949). The pH optima of the other insect proteinases serve to indicate their relationship to trypsin and their differences from cathepsin (note the comparisons made by Balls and Kies 1946).

TABLE 5
pH OPTIMA OF INSECT PROTEINASES

Insect	Reference	Substrate	pH Optimum	pH Optimum, Gelatin Viscosity Method (this paper)
Tineola larvae	Linderstrom-Lang and Duspiva (1936)	Casein, 2 hr.40°C.	9.3	9.8
Lucilia larvae	Hobson (1931*b*)	Collagen, 48 hr. 37°C.	8.5	
Lucilia larvae	Hobson (1931*a*)	Gelatin, 24 hr. 37°C.	7.6	
Periplaneta	Wigglesworth (1928)	Gelatin, 24 hr. 37°C.	7.7	8.4
Periplaneta	Wigglesworth (1928)	Casein, 24 hr. 37°C.	7.1	
Periplaneta	Wigglesworth (1928)	Edestin, 24 hr. 37°C.	8.1	

(*c*) *Oxidation-Reduction Potential*

Most digestive proteases function in a medium in which the redox potential does not differ greatly from the range + 100 to − 100 mv. The observation of Linderstrom-Lang and Duspiva (1936), confirmed in the present investigation, that the oxidation-reduction potential of the midgut of the *Tineola* larva approximates −300 mv. suggested that the protease from this species may be more efficient at this potential. This hypothesis was strengthened by the results of Reiss and Achard (1943) who claimed that the tissue proteinase from *Bombyx* larvae was, in fact, sensitive to changes in oxidation-reduction potential, and further that its maximum hydrolytic effect occurred at about this same figure of −300 mv. The slight effect on *Tineola* proteinase of variations in oxidation-reduction potential over the physiological range indicates that in this particular *Tineola* proteinase is certainly not adapted to the conditions of oxidation-reduction potential under which it functions. A number of authors (Nagai 1939; Sizer 1945; Grob 1949) have reported effects of oxidizing and reducing agents on tryptic proteinases. These experiments have been carried out under a variety of conditions and by different methods and it is not possible to compare directly the results with those reported in this paper. The problems of finding suitable techniques for the study of the effects of differences in oxidation-reduction potential on proteinases have been stressed in Section II above. We have found the method employed in our experiments to be relatively free from errors and our results show no significant influence whatever of the effect of oxidation-reduction potential on the activity of the digestive proteinase from

Periplaneta and *Musca* and relatively small effects on the enzymes from the other insects examined. The effects on papain and trypsin are in the direction expected on the basis of previous work (Sizer 1945; Street 1949).

(*d*) *Enzyme Activity*

During the course of the work reported above, extensive observations have been made on two further aspects of *Tineola* proteinase that conflict with the views of Linderstrom-Lang and Duspiva (1936). These authors report a straight line relationship between enzyme concentration and activity, and this observation was repeated by Duspiva (1936). Some dozens of dilution curves of *Tineola* proteinase have been examined, over a period of 12 months, by a variety of methods for estimating proteinase activity, and in every case a typical Michaelis-Menten dilution curve has been obtained. No explanation can at present be offered for Linderstrom-Lang's finding, but since it does not conform to the theoretical dilution curve, attempts to repeat the result would be of interest.

Again, Linderstrom-Lang and Duspiva (1936) report that the preparations of *Tineola* proteinase were of very considerable activity. In fact, they state that acetone-dried preparations of *Tineola* midgut approach the proteolytic activity of Pancreatin "Merck." Table I will indicate that our preparations were far less active. Preparations made from different instars of *Tineola* larvae and by different methods did not possess activities approaching those reported by Linderstrom-Lang and Duspiva (1936). The considerable activity of acetone-dried caeca of *Locusta,* however, is noteworthy.

The considerations of the previous paragraphs indicate the similarity of the insect enzymes studied, and suggest that the differences observed between them are all of a minor character. It is therefore apparent that the proteinase from *Tineola* is in many respects not especially adapted to its unusual substrate and that the peculiarities of digestion of this species reside in factors other than its protein-digesting enzymes.

V. Acknowledgments

The authors are indebted to Mr. L. A. Marshall for preparing the figures, and to Mr. G. A. McIntyre, Section of Mathematical Statistics, C.S.I.R.O., for statistical analyses.

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