CLVII. ON AN ENZYME FROM BLOW-FLY LARVAE (*LUCILIA SERICATA*) WHICH DIGESTS COLLAGEN IN ALKALINE SOLUTION.

By RALPH PERCIVAL HOBSON.

From the Department of Entomology, London School of Hygiene and Tropical Medicine.

(*Received July 9th, 1931.*)

It is well known that sclero-proteins are not readily digested by the enzymes of vertebrates; both pepsin and trypsin act upon elastin, collagen is very resistant to trypsin but is digested by pepsin, and keratin is not attacked by either enzyme. However, Stankovic, Arnovlzevic and Matavulj [1929] have described a keratinase from the crops of certain birds of prey and Ssadikov [1927] has claimed that a collagenase is present in ox-pancreas and in some commercial samples of pancreatin. According to earlier workers, the pancreatic enzymes are unable to digest collagen [Sahli, 1924].

Lucilia larvae normally develop in meat or carrion, but they are also a serious pest to living sheep, feeding first in the wool and later boring into the tissues. Under suitable conditions the larvae can consume meat entirely, leaving no trace of connective tissue, and, since they cannot ingest solid particles of meat, the digestion of connective tissue must occur outside the body. The larvae secrete a protease which acts in alkaline solution and persists in the excreta [Hobson, 1931]. An investigation, therefore, was made of the action of the excreta on connective tissue proteins. Their action on keratin was also studied, as a keratinase, if present, would explain how the larvae feed in the wool of sheep and penetrate the skin.

Experimental.

The excreta were obtained by placing washed larvae in a filter and allowing water to drip slowly through the filter for several hours. The product is a dark brown alkaline liquid which consists of diluted excreta probably admixed with saliva; its reaction varies from p_H 8·2 to 8·8. Collagen was prepared from the *tendo Achillis* of an ox and from catgut, elastin from the *ligamentum nuchae* of an ox, and keratin from pure wool. In each case the soluble proteins were removed by washing with water and a brief digestion with trypsin.

The action of the excreta on sclero-proteins.

Preliminary qualitative experiments showed that the excreta dissolved small pieces of collagen and elastin completely, the solution showing an increased formaldehyde titration; no digestion, however, occurred with keratin. The relative activity of the excreta on the different proteins and the results obtained with a pancreatin solution are shown in Table I. The digestions

Table I. *The action on sclero-proteins of the excreta from* Lucilia *larvae and trypsin.*

	Excreta preparation	Pancreatin solution	
Collagen*	12	0	mg. dissolved
Elastin	16	25	
Keratin	0	0	
Gelatin	1·5	1·4	Increase in formaldehyde titration in cc.

* *Tendo Achillis.*

were carried out for 48 hours at 37° at p_H 8·0 in the presence of thymol. Proteolytic activity was determined by formaldehyde titrations of gelatin digestions and the action on sclero-proteins by weighing the residues after digestion and estimating the amounts dissolved. The results show that the excreta dissolve collagen, whereas a pancreatin solution of about the same proteolytic strength has no effect. Elastin, however, is digested more readily by pancreatic trypsin than by the protease in the excreta and neither enzyme attacks keratin. Controls, with enzyme solutions which had been heated for 5 minutes at 100°, showed no action.

Attempted separation of collagenase and protease.

Ssadikov [1927] claimed that the collagenase which he found in pancreatic extracts could be separated from the enzyme which digests fibrin (fibrinase) by adsorption with kaolin or charcoal. Kaolin adsorbed the collagenase leaving the fibrinase in solution, while with charcoal the reverse was found. However, with pancreatin solutions Ssadikov obtained different results; after treatment with kaolin the fibrinase was present in both adsorbate and filtrate, the collagenase disappearing, while with charcoal the fibrinase and collagenase were present in both fractions.

These experiments were repeated with the excreta from *Lucilia* larvae. 8 cc. portions of the excreta preparation were shaken at intervals for 30 minutes with 1 g. of the adsorbent, filtered and aliquots of the filtrate tested upon gelatin and catgut, digestion being measured by formaldehyde titration and loss of weight respectively. In addition, the stability of the enzymes in the excreta was studied by measuring the activity of samples which had been kept for different periods at 37°. The results obtained with two preparations of excreta are given in Table II. It is evident that the treatment with charcoal and with kaolin has reduced the activity towards both gelatin and collagen

Table II. *The stability and adsorption of the enzymes in the excreta of* Lucilia *larvae.*

Exp.	Enzyme and treatment	Time of digestion in hours	Action on gelatin (increase in formaldehyde titration in cc.)	Action on collagen (mg. catgut dissolved)
Excreta preparation A:				
1	Unchanged	18	0·95	20
2	Kept 24 hours at 37°	18	0·9	12·5
3	Treated with kaolin	24	0·55	8
4	„ charcoal	24	0·5	4·5
Excreta preparation B:				
5	Unchanged	18	1·45	22
6	Kept 48 hours at 37°	18	1·05	11·5
7	„ 96 „	18	0·80	5·0
8	Treated with kaolin	18	0·95	9·5
9	„ charcoal	18	0·95	5·0

and, although no absolute separation has been effected, comparison of the results shows that differential adsorption has occurred. In Exps. 1, 8 and 9 the digestion of the gelatin has proceeded to the same extent but the amounts of catgut passing into solution are very different (20, 9·5 and 5·0 mg.). The digestion of collagen by the excreta seems therefore to be due to a separate enzyme which will be referred to as collagenase. Both charcoal and kaolin adsorb the collagenase more completely than the protease, charcoal being the more selective. The collagenase also appears to be less stable than the protease in alkaline solution at 37°. The results obtained by adsorbing the excreta of *Lucilia* larvae with kaolin and charcoal are in fair agreement with those of Ssadikov [1927] for pancreatin samples containing collagenase. However, Ssadikov found that kaolin destroyed the collagenase in pancreatin and obtained different results with glycerol extracts of the pancreas.

The origin of the collagenase.

The present author [1931] has already shown that the protease found in the excreta of *Lucilia* larvae is secreted by the mid-gut, but it is possible that the collagenase may be secreted elsewhere or produced by intestinal bacteria. To investigate this question, larvae were starved until the gut was empty, and the salivary glands, mid-guts and hind-guts from 15 larvae were dissected out, each lot being ground up to an emulsion with one drop of glycerol, diluted with 1 cc. of buffer at p_H 8·4 and tested with a small piece of catgut as substrate. Only the mid-gut preparation digested the collagen, and this showed very weak activity compared with the excreta. Furthermore, a preparation of the gut-contents of feeding larvae was found to digest gelatin actively, but not collagen. These results suggest that the collagenase may be produced by bacteria in the hind-gut, but this possibility was disproved by showing the presence of the enzyme in sterile larvae.

The technique of rearing *Lucilia* larvae aseptically will be described in

detail in a later publication; briefly the method used was to sterilise the eggs with 0·1 % mercuric chloride and rear the larvae on autoclaved brain. The residue from the growth of a batch of larvae shown to be sterile was tested by mixing it with an equal volume of buffer at p_H 8·4, adding thymol and catgut and incubating for 2 days at 37°. On examination the catgut showed definite evidence of digestion, being almost completely dissolved.

The collagenase therefore is produced by the larvae and not by bacteria, and there can be little doubt that it is secreted in the mid-gut. The inactivity of the gut-contents from fed larvae is probably due to adsorption of the enzyme by the food, as adding undigested food from the crop has been found to decrease the action of the excreta on collagen. The high ratio of collagenase to protease found in the excreta may be due to the disappearance of the protease, either by its being used up in digestion of the meal or by being partly readsorbed.

The influence of p_H *on the digestion of collagen by the excreta of* Lucilia *larvae.*

To investigate the effect of reaction on collagen digestion, equal lengths of uniform catgut were digested for 48 hours with the same volumes of an excreta preparation at different p_H values. Aliquots of the excreta were titrated with strong acid and alkali to the required reactions by means of a capillary pipette and mixed with equal volumes of buffer of the same p_H. Digestion was measured by estimating the loss of weight and p_H determined colorimetrically at the beginning and end of the experiment, the mean values being taken. The results are plotted in Fig. 1 and show that the optimum reaction for the

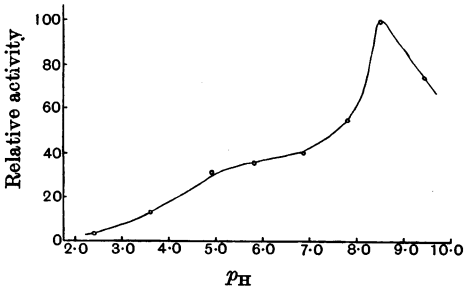

Fig. 1. p_H-activity curve of the digestion of collagen (catgut) by the excreta of *Lucilia* larvae.

collagenase lies between p_H 8·0 and 9·0. This reaction is more alkaline than the optimum found for the digestion of gelatin by the excreta, which was about p_H 7·5 [Hobson, 1931]. The collagenase is well adapted for working in blown meat, in which large amounts of ammonia develop, and the reaction generally lies between p_H 8·0 and 8·5.

Discussion.

The presence of a collagenase in the excreta may play an important part in the growth of *Lucilia* larvae on meat, by digesting the fine strands of connective tissue which surround each muscle fibre and represent to a large extent the structural elements of muscle tissue. The absence of a keratinase is hardly surprising, as this enzyme would only be advantageous in the case of myiasis on sheep, which is exceptional and possibly only a recently acquired habit. When developing in the wool of sheep, *Lucilia* larvae probably feed on the impurities present; if any digestion of keratin occurs, it must be the result of bacterial action.

An enzyme able to digest collagen has been described also by Shinoda [1928] from the gastric juice of the crayfish (*Astacus*). The enzymes from *Astacus* and from *Lucilia* larvae differ in their sensitivity to the reaction; with the gastric juice from *Astacus*, Shinoda found that digestion of collagen occurred over a wide range from p_H 4·0 to 8·0, the actual optimum at p_H 6·7 not being sharply defined; with the excreta from *Lucilia* larvae, the p_H-activity curve (Fig. 1) shows that digestion is limited to a narrow range on the alkaline side of neutrality, the optimum being well marked at about p_H 8·5. Shinoda measured the activity of the gastric juice of *Astacus* at different times during the secretion rhythm against various substrates, but failed to find evidence for the existence of separate proteolytic enzymes. He suggested that, until invertebrate proteases have been separated by modern methods of isolation, reference should be made to "enzyme actions" and not to "enzyme individuals." Shinoda partly based his argument on the fact that the protease from *Astacus*, by digesting collagen in neutral solution, combines to some extent the properties of pepsin and trypsin. However, this is not the case with the protease from *Lucilia* larvae, for adsorption with charcoal decreases the activity of the excreta towards gelatin and collagen to a different extent (Table II) and collagen digestion is, therefore, due to a separate enzyme. Furthermore, Wigglesworth [1928] has shown that the proteolytic enzymes of the cockroach are closely analogous to the corresponding vertebrate enzymes in many respects and was able to separate the tryptase and peptidase fractions by the method used by Waldschmidt-Leitz and Harteneck [1925] for the pancreatic enzymes. There is therefore good evidence that insect proteases include separate individuals which correspond closely to vertebrate trypsin and erepsin.

The reaction products of the collagenase from *Lucilia* larvae could not be determined, as complete separation of the collagenase and protease was not effected. Treatment with dilute acid in the cold or with water at 70° converts collagen into a hydrated form, hydrocollagen, which is digested by trypsin [Ssadikov, 1927], and this may be the end-product of the collagenase from *Lucilia* larvae. It may be noted that the ability of the excreta to digest collagen is not associated with increased action on other resistant proteins;

thus, elastin is digested more readily by trypsin than by the protease from the excreta (Table I).

SUMMARY.

1. The excreta of *Lucilia* larvae contain proteolytic enzymes which digest collagen and elastin but not keratin.

2. The enzyme which digests collagen is produced by the cells of the mid-gut and not by intestinal bacteria, since it occurs in sterile larvae.

3. The excreta digest collagen in alkaline solution, the optimum reaction being at about p_H 8·5. With increasing acidity the activity of the enzyme decreases and almost disappears at p_H 4·0.

4. The separate existence of a collagenase has been concluded from adsorption and stability experiments. The collagenase is less stable than the enzymes acting upon gelatin and is adsorbed to a greater extent by charcoal and kaolin.

I wish to express my appreciation to Dr V. B. Wigglesworth for suggesting this problem and for his active interest in the work. I am indebted to the Empire Marketing Board, for a grant which has financed this work entirely, and to Dr P. A. Buxton, for his interest in this research and hospitality in extending to me the facilities of this Department.

REFERENCES.

Hobson (1931). *J. Exp. Biol.* **8**, 109.
Sahli (1924). *J. Amer. Med. Assoc.* **82**, 51.
Shinoda (1928). *Z. vergl. Physiol.* **7**, 323.
Ssadikov (1927). *Biochem. Z.* **181**, 267.
Stankovic, Arnovlzevic and Matavulj (1929). *Z. physiol. Chem.* **181**, 291.
Waldschmidt-Leitz and Harteneck (1925). *Z. physiol. Chem.* **149**, 203.
Wigglesworth (1928). *Biochem. J.* **22**, 150.