XXII. DIGESTION IN THE COCKROACH.

III. THE DIGESTION OF PROTEINS AND FATS.

By VINCENT BRIAN WIGGLESWORTH.

From the Department of Medical Entomology, London School of Hygiene and Tropical Medicine, W.C. 1.

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This paper concludes a comparative study of the digestive enzymes of the cockroach. In the first and second papers of the series [Wigglesworth, 1927, 1, 2] it has been shown that the crop contents of this insect are normally rendered acid (p_H 4·6–6·0) by the action of micro-organisms upon the carbohydrates in the food, and that the enzymes concerned in carbohydrate digestion are active over this range of p_H, but, in other respects, resemble the corresponding enzymes from other sources. When the food consists of protein alone, the reaction of the crop is about 6·2. The present paper deals with the enzymes concerned in the digestion of proteins and fats.

Proteolytic enzymes.

Preliminary qualitative tests gave results in agreement with those of Swingle [1925] and Abbott [1926], who state that the proteolytic enzymes of the cockroach are secreted only in the mid-gut and that pepsin-like enzymes are absent, digestion being most active in weakly alkaline solution. This is well seen in Table I which shows the digestion of fibrin at different p_H values. Blood-fibrin was ground up into particles about the size of coarse sand, stained with Congo red by the method of Roaf [1909] and preserved in glycerol. For the experiment shown, the fibrin was washed, squeezed dry and weighed out in 0·075 g. quantities. These were mixed with 0·7 cc. of buffer solution (Clark and Lubs) and 0·1 cc. of a glycerol extract from the mid-gut of *Periplaneta americana* or 0·1 cc. of pancreatin in 50 % glycerol, and shaken in a water-bath at 37° for 24 hours. Control experiments with boiled enzyme preparations showed that "fibrolysis" [Long and Barton, 1914] played no part in the digestion observed.

Table I.

	p_H	1·1	2·2	3·0	4·0	5·0	6·0	7·0	8·0
Digestion after 24 hrs.	Cockroach protease	–	–	–	±	+	+ +	+ + +	+ +
	Pancreatic trypsin	–	–	–	–	±	+	+ +	+ + +

At the end of this experiment the p_H in the tubes at 2·2 and 3·0 was brought back to 7·0 by the addition of alkali. Twenty-four hours later there was well-marked digestion in both tubes. Like pancreatic trypsin the cockroach protease is inactivated, but is not destroyed, by moderate acidity. Like trypsin again the enzyme was found to be completely destroyed by keeping at about p_H 12 at 37° for 30 minutes.

These results suggested that the protease of the cockroach is closely similar to mammalian trypsin; for although the former appeared to be rather more active in the acid direction, yet the maximal activity is well to the alkaline side of the isoelectric point of the protein substrate; and it is therefore clear that the enzyme does not resemble in this respect the plant proteases of the papain type [Willstätter and Grassmann, 1924]. A comparison of pancreatic trypsin and cockroach protease was therefore undertaken.

Comparison of pancreatic trypsin and cockroach protease.

(i) *Products of digestion of caseinogen.* A crude glycerol extract of cockroach mid-gut was incubated at 37° with 10 % caseinogen at about p_H 7·5 with the addition of toluene. At the end of 10 days the mixture was heated on a boiling water-bath for an hour and filtered hot. The filtrate gave an intense bromine reaction for free tryptophan and after evaporating to small bulk and allowing to stand 24 hours, crystals of leucine and tyrosine separated out. These results are the same as those obtained with pancreatic trypsin.

(ii) *The relative production of "free acidity" and "formaldehyde acidity" during digestion.* Some years ago Mr S. W. Cole of the Biochemical Laboratory, Cambridge, observed an interesting distinction between peptic and tryptic digestion of proteins as followed by the formaldehyde titration of Sørensen. These observations were communicated to the Biochemical Society but have never been published, and I am indebted to Mr Cole for permission to reproduce here his original curves (Figs. 1 and 2).

When gelatin (or other protein) is acted upon by proteolytic enzymes, the quantity of standard alkali required to titrate the mixture to an arbitrary p_H (say, 8·4) is found to increase as digestion proceeds. This may be provisionally termed "free acidity." If this mixture (at p_H 8·4) be tested with neutral formaldehyde, the reaction becomes acid, due to the formation of methylene compounds, and a further amount of standard alkali is required to bring the mixture back to p_H 8·4. This may be termed "formaldehyde acidity."

Figs. 1 and 2 show the "free acidity" and "formaldehyde acidity" produced during the digestion of gelatin by trypsin and pepsin respectively. It will be seen that in the case of trypsin, during the initial stages of digestion, the increase of the "free acidity" is rather more rapid than that of the "formaldehyde acidity," but that, whereas the former soon falls short and remains constant, the latter continues to increase. On the other hand, during

peptic digestion the "free acidity" far outstrips the "formaldehyde acidity." The same result is obtained in the digestion of albumin and caseinogen.

Although the explanation of this phenomenon is quite obscure (Cole inclines to the view that it indicates a chemical difference in the type of linkage which is broken by the two enzymes) yet it remains a very interesting empirical means of differentiating the two types of protein break-down.

Fig. 3 shows the digestion of albumin by the protease of the cockroach. It is clear that this is of the tryptic type.

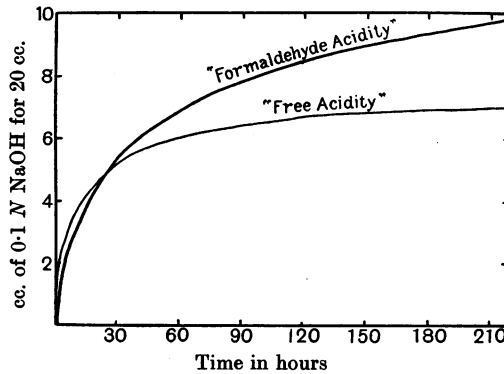

Fig. 1. Digestion of 4·5 % gelatin by trypsin at p_H 8·1. (Cole.)

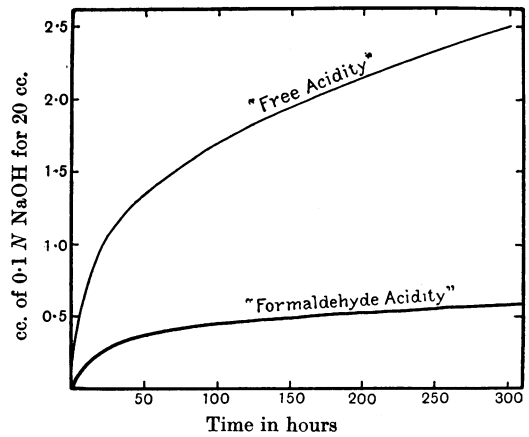

Fig. 2. Digestion of 4·5 % gelatin by pepsin at p_H 1·4. (Cole.)

(iii) *Effect of salts.* While trypsin is not strikingly affected by the presence of salts, it is well known that papain is strongly activated by cyanides, and Ringer and Grutterink [1926] have shown that this enzyme is activated also by phosphates.

For the investigation of the effect of salts, the rate of clearing of a suspension of coagulated albumin has been used as a test of proteolytic activity. A 0·5 % solution of serum-albumin was placed in a boiling water-bath and

traces of dilute hydrochloric acid added very cautiously until a deep cloud, without any flocculation, was produced. 0·5 cc. quantities of this suspension were measured into uniform small test-tubes (100 by 6 mm.) and the volume made up to 1·0 cc. with the enzyme and other reagents under investigation. A standard tube was prepared by taking 0·25 cc. of the albumin suspension and making up to 1·0 cc. with distilled water. The time required for the degree of clearing in the experimental tubes to equal that of the standard, gave a measure of the enzyme activity. The opacity of the two tubes could be compared very accurately by looking through each at two identical words printed in small type on white paper.

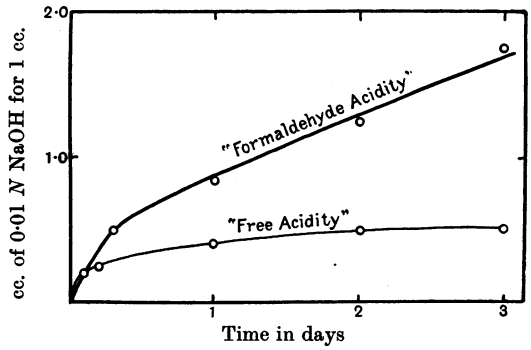

Fig. 3. Digestion of 3 % albumin by cockroach protease at p_H 7·6.

By this means the activity of cockroach protease at p_H 6·0 in the presence of phosphate and phthalate buffers (about 0·04 *M*) was compared and found to be identical; similarly at p_H 8·0 in the presence of phosphate and borate buffer.

Hydrocyanic acid had no activating effect but, as in the case of trypsin, caused some inhibition. This is shown in Table II.

Table II.

Concentration of KCN g. per 100 cc.	Relative velocity at p_H 7·0	
	Trypsin	Cockroach protease
0	100	100
0·2	87	86
0·5	70	60

(iv) *The p_H-activity curves with different substrates.* The preliminary experiments with coloured fibrin, described above, seemed to indicate that the range of activity of cockroach protease extends further in the acid direction than that of pancreatic trypsin. Experiments made to investigate this point more carefully are given in Figs. 4, 5, 6 and 7.

Fig. 4 shows the digestion of serum-albumin. This was carried out by the opacity method described in the last section, after it had been shown that the initial opacity was not affected by the range of p_H used and that flocculation did not occur until the p_H was below 6·0.

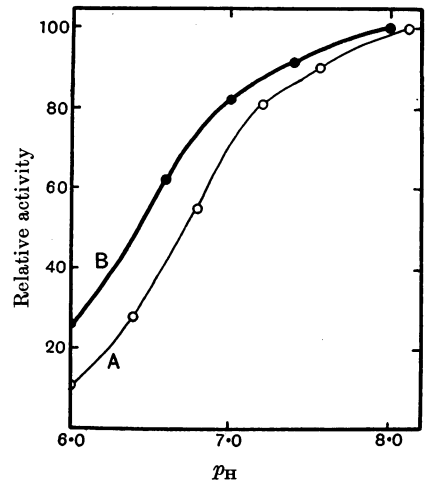

Fig. 4. p_H-activity curves of digestion of serum-albumin (0·5 %). A, Trypsin; B, Cockroach protease.

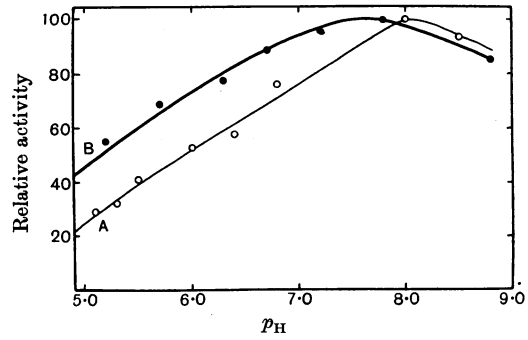

Fig. 5. p_H-activity curves of digestion of gelatin (5 %). A, Trypsin; B, Cockroach protease.

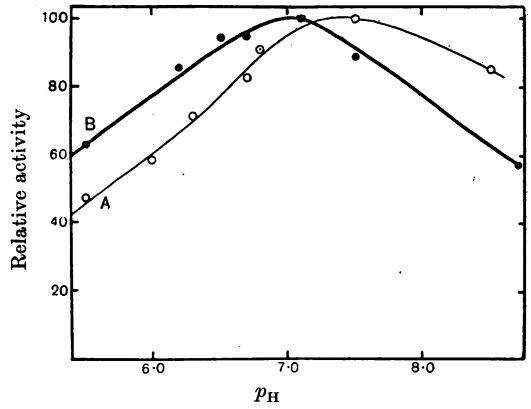

Fig. 6. p_H-activity curves of digestion of caseinogen (5 %). A, Trypsin; B, Cockroach protease.

For the observations on gelatin, caseinogen and edestin (Figs. 5, 6 and 7) the formaldehyde titration was used. The experimental mixtures consisted of 0·1 cc. of enzyme, 0·4 cc. of an appropriate buffer and 0·5 cc. of substrate. After the addition of toluene the mixtures were shaken in a water-bath at 37°. At the start and at the end of 24 hours, 0·1 cc. of each mixture was measured into 5 cc. of distilled water and the formaldehyde titration carried out with $N/100$ NaOH. The p_H was determined colorimetrically after diluting with 8 volumes of distilled water. During the course of the experiment there was an increase in acidity which varied between p_H 0·1 and 0·3. The figure used in the construction of the curves is the mean between those obtained at the beginning and at the end of the experiment.

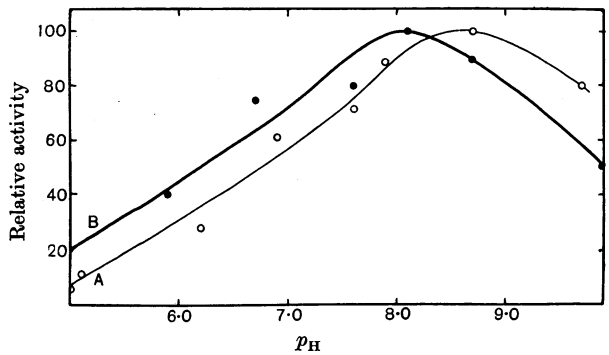

Fig. 7. p_H-activity curves of digestion of edestin (2 %). A, Trypsin; B, Cockroach protease.

The experiments show consistently that with a given protein the range of activity of the cockroach protease extends further in the acid direction than is the case with pancreatic trypsin.

This result is of considerable interest; for Northrop [1922] maintains that the form and position of the p_H-activity curve of trypsin is determined solely by the titration curve of the substrate protein. Were this the case, it would follow that, under the same experimental conditions, the p_H-activity curves of trypsins from different sources should be identical. The above experiments, however, show that the trypsin of the cockroach is more active in the acid range than is pancreatic trypsin and they suggest that, as is the case with the enzymes acting upon carbohydrates, the p_H-activity curve is determined in part at least by the nature of the enzyme or, as is the case with lipolytic enzymes, by the nature of associated substances. On the other hand the results are in general agreement with those of Northrop, for it will be seen that it is proteins such as gelatin and caseinogen, whose dissociation curves are further in the acid zone, which are the more readily digested in acid solution; while edestin with a less acid isoelectric point is less readily digested at the lower p_H.

Erepsin.

Swingle [1925], on the grounds that an extract from the gut of the cockroach would cause an increase in the formaldehyde titration of a solution of peptone, and would produce, in this digest, nitrogenous compounds not precipitated by phosphotungstic acid, states that an erepsin is present in the alimentary tract of this insect. The work of Waldschmidt-Leitz and Harteneck [1925] has resulted in a more precise definition of erepsin (peptidase) as an enzyme capable of hydrolysing only dipeptides and tripeptides, substances upon which trypsin is without any action. There is in fact no overlap in the range of action of the two enzymes. It seemed desirable therefore to investigate again the peptidase of the cockroach.

For qualitative experiments, as advocated by Pfeiffer and Standenath [1925], the dipeptide, glycyltryptophan, has been employed. A solution of this dipeptide ("Fermentdiagnostikum") was kindly supplied by Messrs Bayer Products Ltd.

The mid-gut of a single *P. americana* was ground up in 1·0 cc. of water with a little thymol. 0·1 cc. of this emulsion was mixed with 0·4 cc. of water and 0·1 cc. of glycyltryptophan and incubated at 37°. A similar mixture containing boiled emulsion was used as a control. At intervals, 0·1 cc. was withdrawn, mixed with a drop of 5 % acetic acid, and minute traces of bromine water added. The test mixture showed a faint pink at the end of 2 hours and an intense pink after 24 hours. The control showed no change. It is clear that the gut of the cockroach contains a true erepsin. Further experiments conducted in the same manner showed that, as was shown by Swingle [1925] to be the case with the other digestive enzymes, the erepsin is actually secreted into the gut contents.

An attempt was made to separate this enzyme from the trypsin above described. For this purpose the technique of Waldschmidt-Leitz and Harteneck [1925] was followed. Sixteen cockroaches (*P. americana*) were dissected and the mid-guts ground up with thymol and glycerol, made up to 16 cc. with 50 % glycerol and filtered through cotton wool. This extract gave positive tests for trypsin and for peptidase. 8 cc. of this extract, with 0·5 cc. of phthalate buffer, p_H 4·6, were shaken up with 1·5 cc. of freshly prepared aluminium hydroxide[1], allowed to stand 2 minutes and centrifuged. This procedure was repeated twice upon the supernatant fluid which was then brought to about p_H 7·0 and will be referred to as preparation A. The precipitate of aluminium hydroxide in the original mixture was extracted for 2 hours with 8 cc. of $N/25$ ammonia in 18 % glycerol. The mixture was then centrifuged and the supernatant fluid brought to about p_H 7·0 (preparation B).

Tables III and IV show the results of tests applied to the resulting preparations.

[1] 6·8 g. of aluminium sulphate were dissolved in 40 cc. of water and $N/5$ ammonia added to 100 cc.

Table III. *Mixtures:* 0·1 *cc. of enzyme preparation* + 1·0 *cc. of calcified milk diluted* 1 *in* 10.

	Preparation A		Preparation B	
	Test mixture	Boiled control	Test mixture	Boiled control
9.15 a.m.	—	—	—	—
9.20 „	Clotting complete	—	—	—
10.45 „	„ „	—	Clotting beginning	—
11.15 „	„ „	—	„ complete	—

Table IV. *Mixtures:* 0·1 *cc. of enzyme preparation* + 0·4 *cc. of phosphate buffer* p_H 6·6 + 0·1 *cc. glycyltryptophan.*

	Preparation A		Preparation B	
	Test mixture	Boiled control	Test mixture	Boiled control
9.45 a.m.	—	—	—	—
6.0 p.m.	—	—	Faint pink	—

It is clear from these experiments that a partial separation of the enzymes has been accomplished. Preparation A containing most of the trypsin and B containing most of the peptidase; *i.e.* in their adsorption by aluminium hydroxide they behave like the trypsin and erepsin of the pancreas as studied by Waldschmidt-Leitz and Harteneck.

Few comparative studies on peptidases have been made. Dernby [1917] compared yeast erepsin and gut erepsin and showed that they have the same p_H optimum (7·8) but differ in the effect of neutral salts. Yeast erepsin is unaffected by a salt concentration up to 0·5 *M* while gut erepsin is markedly depressed by 0·02 *M* salt concentration.

For the investigation of the p_H range of the cockroach erepsin, glycylglycine has been used as substrate and the digestion followed by formaldehyde titration. As described by Dernby the solution becomes more alkaline as digestion proceeds. Under the conditions of experiment this caused an increase in p_H of 0·5 in the mixture showing the greatest change. The figures used in the construction of the curve (Fig. 8) are the means of the p_H values, determined colorimetrically, at the beginning and at the end of the experiment 24 hours later. For the experiment given in Fig. 8 the mid-gut of a single *P. americana* was ground up in 1 cc. of water with thymol. The mixtures consisted of 0·1 cc. of this enzyme solution, 0·3 cc. of buffer and 0·4 cc. of 2 % glycylglycine. Owing to shortage of material this experiment was the only one performed. It shows an optimal zone around p_H 8·5.

Lipase.

If the cockroach be kept without food after a meal of carbohydrates, the crop is usually empty again at the end of 48 hours; but this is not the case after the ingestion of fat. Sandford [1918] states that a large meal of fat may remain in the crop of *P. americana* for 2 months, though Abbott [1926] working with *P. australasiae* failed to observe its persistence for such

long periods. These observations suggest that either the cockroach gut is poor in lipase, or the enzyme is acting in an unsuitable medium.

An active lipase was discovered by Plateau [1873] in extracts from the gut of the cockroach, and since that time there has been a considerable controversy as to the distribution of lipase and the seat of absorption of fat. The subject is reviewed by Abbott [1926]. Sandford [1918] and Swingle [1925] assert that the crop is of equal importance to the mid-gut in the secretion of lipase; but Abbott [1926], using *P. australasiae*, has shown that, as is the case with the enzymes acting upon carbohydrates, crop lipase is due entirely to regurgitation from the mid-gut.

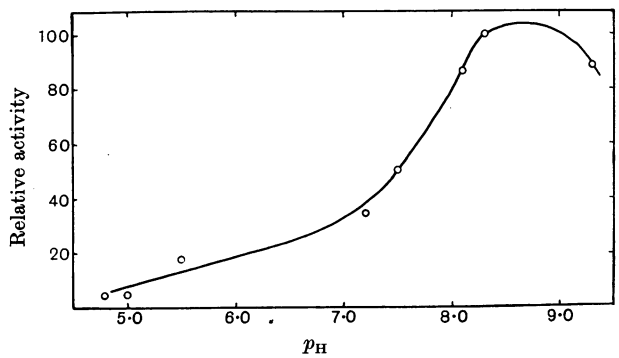

Fig. 8. p_H-activity curve of digestion of glycylglycine by cockroach erepsin.

Table V shows how the relative lipolytic activities of the crop and mid-gut of *Blatella germanica* are affected when the crop is washed out by giving water but no food for a fortnight. In each case the crops and mid-guts of a number of insects were separated, made up to an equal volume with water, and the lipolytic activity of the two resulting extracts compared as described below.

Table V.

Notes	Relative activity	
	Crop	Mid-gut
Freshly caught *B. germanica*	67	100
Same batch after having water alone for 15 days	18	100

These results support those of Abbott; but it is clear that lipase is removed from the crop with more difficulty than are the carbohydrate enzymes; and the possibility remains that some of the residual lipase demonstrated in Table V is due to intracellular enzyme in the cells of the crop, which would not be discharged into the gut.

Abbott has also shown conclusively that whereas the chitinous lining of the crop renders it quite impermeable to sugars and other substances in aqueous solution, it is readily permeable *in vitro* to fats and fat solvents, and that in the living animal, undigested fat passes through the chitinous lining

and is taken up by the cubical cells of the crop wall. Any deficiencies in the digestion of fat may therefore perhaps be compensated to some extent by the absorption of undigested material.

The comparative study of lipases is rendered difficult and unsatisfactory by the great extent to which their properties are affected by associated substances, so that a lipase from a given source may vary greatly in its properties depending upon its degree of purification. This applies to the optimum range of p_H [Willstätter, Haurowitz and Memmen, 1924], to the effect of bile salts [Willstätter and Memmen, 1924] and to the relative activity upon lower esters and the glycerides of the higher fatty acids [Platt and Dawson, 1925].

No great significance therefore can be attached to the experiments given here, where the lipase in a crude aqueous emulsion of cockroach mid-gut has been compared with an aqueous solution of pancreatin.

Methods. Enzyme preparations from the cockroach consisted of the mid-gut of a single *P. americana* freshly ground up with a little thymol in 1 cc. of water to give a uniform emulsion. Pancreatic lipase was obtained by dissolving pancreatin in water and neutralising to about p_H 7·0.

Lipolytic activity has been measured by the production of acid from ethyl butyrate. 0·2 cc. of enzyme was mixed with 0·8 cc. of buffer solution (Clark and Lubs) in a small test-tube, and 0·1 cc. of neutralised ethyl butyrate added. 0·1 cc. of this mixture was withdrawn, added to 5 cc. of distilled water and titrated with $N/100$ NaOH to p_H 9·0 using thymol blue as indicator. The mixtures for comparison were shaken side by side in a water-bath at 37° and the titration repeated at the end of one or two hours. Under these conditions the change in p_H during the experiment seldom exceeded 0·4. In a few experiments a 25 % emulsion of olive oil was used in place of the ethyl butyrate.

(i) *Effect of bile salts.* There is considerable uncertainty as to the effect of bile salts upon lipase. The digestion of true fats is said to be increased by the improved emulsification in the presence of bile salts; and in addition, bile salts are believed by Platt and Dawson to have a specific chemical effect in stimulating lipase, as evidenced in the hydrolysis of ethyl butyrate. Abbott observed a definite increase in the breakdown of olive oil by the lipase of the cockroach in the presence of ox bile.

The results obtained with sodium glycocholate (0·1 %) and fresh aqueous extracts of cockroach mid-gut have been variable and often uncertain; but usually there has been increased action upon both olive oil and ethyl butyrate. The same variability occurs in experiments with pancreatic lipase.

(ii) *Effect of phosphates.* Platt and Dawson hold that phosphates have a specific activating effect upon pancreatic lipase, independent of their influence as buffers.

This observation has been confirmed in the case of pancreatic lipase but could not be demonstrated with the crude cockroach lipase. The mixtures

used are given in Table VI. At the end of the experiment the p_H in the phosphate and phthalate mixtures was shown to be the same.

Table VI. 0·1 *cc. enzyme* + 0·9 *cc. buffer*, p_H 6·0, + 0·1 *cc. neutralised ethyl butyrate.*

		Increase in acidity at end of 2 hrs. N/100 NaOH for 1 cc.	
Pancreatic lipase.	Phosphate buffer	(*a*) 1·8	(*b*) 1·7
,,	Phthalate ,,	(*a*) 1·3	(*b*) 1·3
Cockroach lipase.	Phosphate ,,	(*a*) 1·0	(*b*) 1·1
,,	Phthalate ,,	(*a*) 1·1	(*b*) 1·1

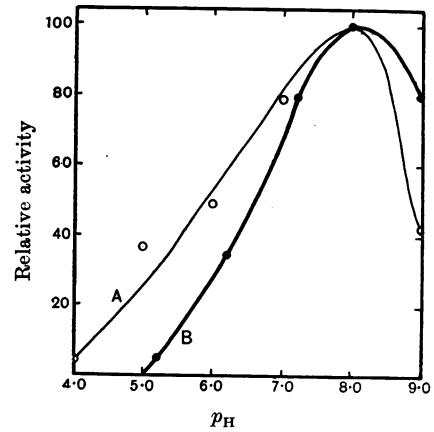

Fig. 9. p_H-activity curve of hydrolysis of ethyl butyrate by pancreatic lipase (A) and cockroach lipase (B).

(iii) p_H-*activity curve.* Fig. 9 shows the p_H-activity curve for pancreatic lipase compared with that for the crude extract of cockroach gut. The curve (A) for pancreatic lipase agrees fairly well with that obtained by Platt and Dawson for a crude glycerol extract of pig's pancreas. But for the reasons stated in the introductory paragraph on lipase, the precise position of these curves is of little significance. One fact, however, is clear; the lipase of the cockroach is completely inactive at p_H 5·0 and is therefore singularly ill-adapted to function in the crop of the living insect where the p_H 24 hours after a meal of fat varied in several insects from 4·8 to 5·2.

Summary.

1. The proteolytic enzymes of the cockroach consist of a tryptase and a peptidase. Peptase is absent.

2. The tryptase resembles pancreatic trypsin in (*a*) the products of digestion, (*b*) the relative production of "free acidity" and "formaldehyde acidity" during digestion, (*c*) the effect of salts and (*d*) in acting upon proteins only on the alkaline side of their isoelectric point.

3. The two enzymes differ in that with a given protein the range of activity of the cockroach protease extends further in the acid direction. The theoretical significance of this fact is discussed.

4. The peptidase is present in the intestinal secretion. It has been partially separated from the tryptase by adsorption. From a single experiment it appears to have an optimal zone of activity about p_H 8·5.

5. Lipase, as present in a crude emulsion of the cockroach gut, is usually, but not constantly, activated by sodium glycocholate; it is not activated by phosphates apart from their influence as buffers, and it shows an optimal p_H zone around 8·0. It is almost inactive at the normal reaction of the crop.

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