THE ACTION OF PROTEOLYTIC ENZYMES ON THE ANTITOXINS AND PROTEINS IN IMMUNE SERA.

III. FURTHER STUDIES ON ENZYME SYSTEMS WHICH SPLIT THE ANTITOXIN MOLECULE.

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IN earlier papers (Pope, 1938, 1939*a*, *b*) results were presented showing the action of proteolytic enzymes on antitoxic sera. Additional work had been carried out in 1939 but was not completed owing to the outbreak of war, and only recently has it been possible to complete and extend this work.

The purpose of the present paper is to show in more detail the manner in which enzymes that split the antitoxin molecule act, and to present evidence which suggests that in pepsin, trypsin, papain, rennin, etc., there is present a special enzyme which has this function.

EXPERIMENTAL.

Methods.—Protein determinations and antitoxin estimations were made as described previously (Pope, 1939). For most of the work diphtheria antitoxic sera were used in the form of oxalated plasma, but in some cases a special insoluble substrate, described in detail later, was used. The enzymes employed are dealt with in detail as the work with them is described.

Optimal pH for Enzyme Action.

Experiments with pepsin were carried out in the following manner : Diphtheria antitoxic plasma, diluted with two volumes of water, was adjusted to pH values of 3·2, 3·6, 4·0 4·5, 5·0, 5·5, 6·0, 6·5 and 7·0, and to each 100 ml. 20 mg. of pepsin (1/3000 activity) was added. These samples were then heated to 37° for 24 hours, after which they were adjusted to pH 4·3 and 14 per cent ammonium sulphate (w/v) added. After filtration the antitoxin content of each filtrate was determined. In order to determine whether the antitoxin had the property of heat stability previously described (Pope, 1938, 1939) portions of the filtrates were heated to 60° for one hour. This heating caused precipitation of protein, and after filtration the antitoxin values were again determined. Results for this experiment are shown in Fig. 1. The antitoxin values in the filtrates, before heating, show an obvious effect of pH ; at values more acid than pH 5·0 there is a rapid fall in the antitoxin values. After the heat treatment the curve shows an optimal pH value for survival of the antitoxin at about 3·75 ; at pH 4·5 only 5 per cent of the antitoxin remains after heat treatment.

The action of pepsin is, as might be expected, restricted to the acid side, and it was therefore a matter of some interest when it was found that crude trypsin behaved similarly.

Fig. 2 shows results for an experiment in which Liq. Trypsin Co. (Allen & Hanbury's) was used. Conditions generally were as for the previous experiment except that the pH range was 3·2, 3·4, 3·8, 4·2, 4·5, 5·0, 6·0, 7·0, 5 per cent Liq. Trypsin Co. added to each, and the solutions were held for 4 days at 20° before

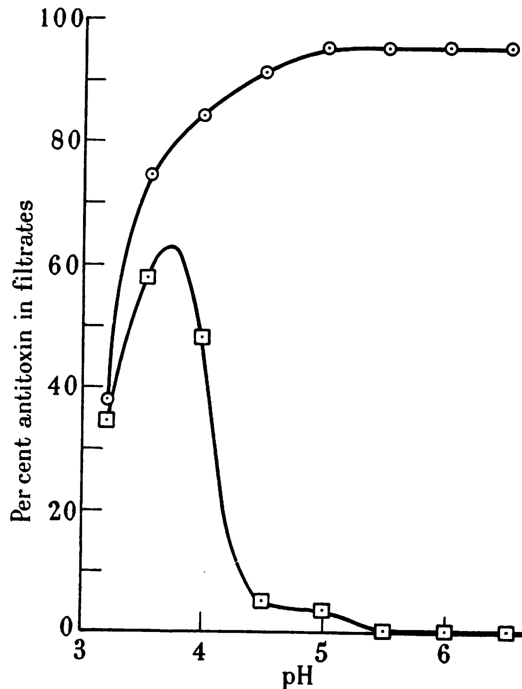

Fig. 1.—The action of pepsin (20 mg./100 ml.) on diphtheria antitoxin at varying pH values for 24 hours at 37°. All samples made to 14 per cent w/v ammonium sulphate and pH to 4·3 after incubation.

⊙——⊙ Per cent antitoxin in filtrates before heating.

⊡——⊡ Same after heating for 1 hr. at 60°.

adjusting to pH 4·3 and adding 14 per cent ammonium sulphate (w/v). After filtration the antitoxin value and protein content were determined (curves A and B, Fig. 2). Portions of each filtrate were heated to 60°, but in this experiment the time of heating was limited to 20 minutes. After filtration the antitoxin value and protein content were again determined (curves C and D, Fig. 2). Once again the maximal antitoxin stability on heating to 60° was at about pH 3·75. Curve D also shows that the soluble protein in the heated filtrates follows that of the antitoxin and is maximal at the same pH value. Experiments similar in nature were also carried out with crude papain (without activation) and with commercial rennin powder. In each case the pH for optimal activity was the same as for pepsin.

These results suggested that it would be a matter of interest to examine crystalline enzymes, and samples of crystalline trypsin and chymotrypsin were obtained (our thanks are due to Dr. J. Northrop for these materials). A modified method was used for the examination of these enzymes. The diphtheria antitoxic plasma, diluted with two volumes of water, was adjusted to pH 3·8, and either crystalline trypsin or chymotrypsin added in the quantities shown in Fig. 3. These amounts relate to the enzymes as supplied, and inert salts account for about 50 per cent of the weight; the actual quantities of enzyme proteins are therefore

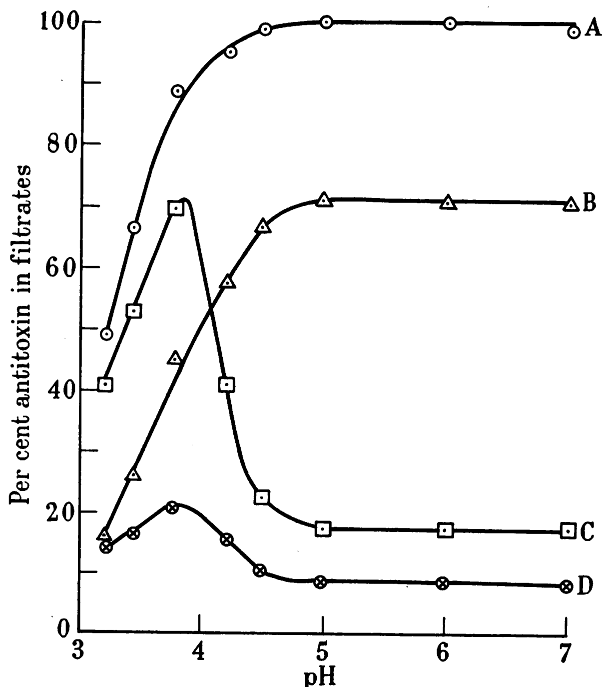

Fig. 2.—The action of crude trypsin on diphtheria antitoxin at varying pH values (5·0 ml. A & H trypsin/100 ml.). Kept for 4 days at 18°; then 14 per cent w/v ammonium sulphate and all adjusted to pH 4·3. Part filtered and remainder heated to 60° for 20 minutes.

A—Per cent antitoxin in filtrates (non-heated)
B—Per cent protein in filtrates (non-heated)
C—Per cent antitoxin in filtrates (heated)
D—Per cent protein in filtrates (heated)

about half those shown. After heating to 37° for 24 hours, 14 per cent (w/v) ammonium sulphate was added and the mixtures filtered. From the results in Fig. 3 it will be seen that in the absence of enzyme only a small percentage of the original antitoxin was found in the filtrates (about 10 per cent). Crystalline chymotrypsin was active in liberating antitoxin from the insoluble substrate, while crystalline trypsin was considerably less active. The characteristic increase in soluble protein is also shown. The antitoxin splitting activity of these crystalline enzymes at pH 3·8 was surprising, since this pH is far removed from that at which they are optimally active against substrates such as denatured haemoglobin.

We have also examined enzyme extracts prepared from ox and sheep pancreas. Using ox pancreas a preparation was made in the following way :

Minced ox pancreas (2000 g.) was diluted to 10 litres with water and allowed to stand for 24 hours at 4° ; 10*N* HCl was then added to adjust the pH to 3·6, and the product filtered. Each litre of filtrate was precipitated by adding 250 g. of ammonium sulphate and the precipitate discarded. The filtrate was again precipitated by adding a further 250 g./litre ammonium sulphate and the precipitate collected by filtration. It was dissolved in water to give a volume equal to 1/10 of the original. This solution was found to contain 0·37 per cent of protein.

Using this enzyme preparation, tests for enzyme activity were made as described for crystalline trypsin and chymotrypsin. The results obtained are

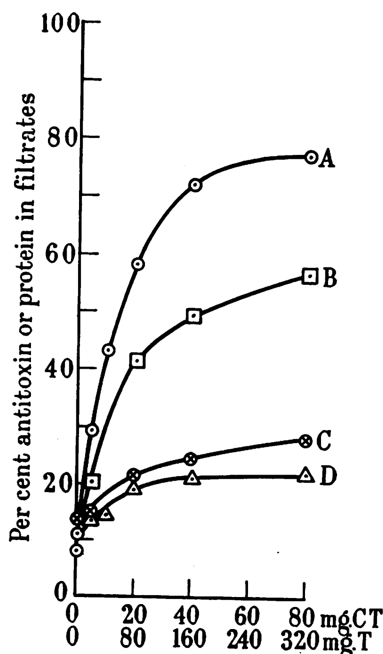

Fig. 3.—The action of crystalline chymotrypsin (CT) and trypsin (T) (Northrop) on diphtheria antitoxin at pH 3·8–24 hrs. at 37°. Liberation of antitoxin by increasing amounts of chymotrypsin A and trypsin B. Liberation of protein by increasing amounts of chymotrypsin C and trypsin D.

shown in Fig. 4. From these results it is clear that very much smaller quantities of enzyme protein from pancreas were needed for the liberation of antitoxin from the insoluble substrate than was the case with either crystalline trypsin or chymotrypsin. This preparation was not a pure enzyme ; it is therefore clear that even smaller quantities of pure enzyme would be required to liberate the antitoxin from the substrate. From this observation it appeared that the crystalline trypsin and chymotrypsin might be considered as having, as a contamination, a small amount of another enzyme which was active at pH 3·8 against the antitoxin substrate.

A further study was therefore made on this enzyme, using sheep pancreas which was available in considerable quantity. The isolation of the enzyme fraction and the methods used for assay are described below.

Isolation of the Antitoxin-splitting Enzyme from Sheep Pancreas.

Frozen sheep pancreas (1000 g.) was minced and extracted with cold N/4 H_2SO_4 (4·0 l). After straining through muslin the liquid was adjusted to pH 6·0 and re-filtered with the aid of Hyflo Supercel on a Buchner funnel. The

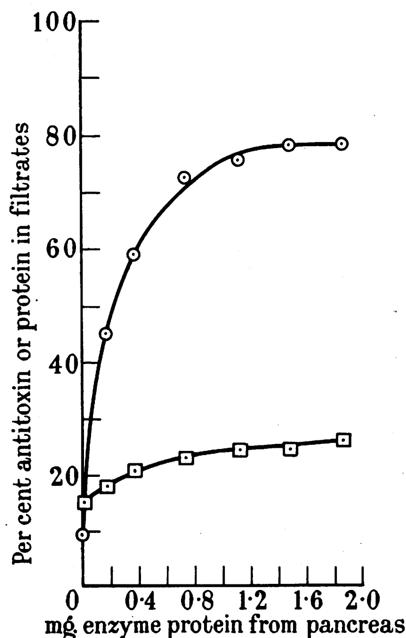

FIG. 4.—The action of an enzyme isolated from pancreas on diphtheria antitoxin at pH 3·8 and 24 hours at 37°. ⊙ per cent antitoxin and ⊡ per cent protein solubilised by increasing amounts of enzyme.

protein in the filtrate was precipitated completely with ammonium sulphate, and after separation by filtration, dissolved in water, dialysed to remove excess ammonium sulphate, and the volume then made to 500 ml. After adjusting the pH to 4·0 the protein was precipitated fractionally to yield six fractions as shown in Table I. Each fraction, after dialysis, was adjusted to a volume of 100 ml. and the protein content of each determined. The total protein present in the six fractions was 1·753 g. derived from 1000 g. of pancreas.

For the purpose of assaying the enzyme activity of these fractions the following method was used: Diphtheria antitoxin plasma was fractionated with ammonium sulphate and the protein precipitating between 1/3 and 1/2 saturation was isolated. The antitoxic protein was dissolved in water (giving a value of about 120 units per ml.), 8 per cent (w/v) ammonium sulphate added and the pH adjusted to 3·8. After heating to 55° for 1 hour the antitoxin became

insoluble and tests on a filtered portion showed the filtrate to be antitoxin-free. This substrate when acted upon by a slight excess of a suitable enzyme (pepsin or trypsin fraction) gave a maximal liberation of 80 units/ml. of antitoxin. As an arbitrary enzyme unit we selected the amount of enyzme which would liberate 30 units of antitoxin from this substrate in 24 hours at 37°. This was on the linear part of the curve relating the square root of the enzyme concentration with the units of antitoxin liberated under these conditions. Table I shows the results obtained in this experiment.

TABLE I.—*The Fractionation with Ammonium Sulphate of the Antitoxin-splitting Enzyme from Sheep Pancreas.*

Fraction.	Ammonium sulphate g./litre.	Volume of enzyme ≡ 1 unit. ml.	Enzyme units per ml.	Per cent of total enzyme present.
C1	0–100	0·0484	20·6	1·35
C2	100·150	0·0484	20·6	1·35
C3	150·200	0·0056	178·5	11·6
C4	200–250	0·0009	1111·0	72·5
C5	250–300	0·0056	178·5	11·6
C6	300–350	0·0484	20·6	1·35

The volume of enzyme solution equivalent to 1 unit was determined from a number of experiments in which the amount of antitoxin liberated from the substrate was both slightly more and slightly less than 30 units per ml., and the volume required to liberate 30 units found by interpolation. It will be seen that most of the enzyme was precipitated between 200 and 250 g. of ammonium sulphate per litre, and this was confirmed in a series of experiments. Many attempts were made to isolate the enzyme present in this fraction in crystalline form but without success.

Since the splitting of the antitoxin molecule gives no information about other enzymes present in this fraction we have examined its activity on haemoglobin at a series of pH values. The method used (Pope, Fenton and Jones, unpublished) is based on that of Anson (1938), except that crystalline horse haemoglobin (undenatured) was used as substrate, and the method used to produce the colour in the digestion products with the Folin-Ciocalteu phenol reagent (Folin and Ciocalteu, 1927) was modified in order to obtain more stable colours. A detailed account of this method and work on the fractionation of enzymes will be published elsewhere. Our choice of undenatured crystalline haemoglobin as substrate was made because the undenatured haemoglobin is less readily attacked by trypsin and chymotrypsin, and we wished to determine the degree of proteolysis in the acid pH zone. Fig. 5 shows the results obtained on examination of the active enzyme fraction in this way. The haemoglobin substrate was added to buffer solutions, and the pH values adjusted in steps of 0·25 pH unit across the range 2·0 to 9·0. To each mixture was added 0·5 ml. of a 1/20 dilution of the enzyme fraction C4 (see Table I); digestion was allowed to proceed for 4 hours at 40°. Excess substrate was then precipitated with trichloracetic acid (5 per cent final concentration), and after standing for at least 4 hours (or overnight) the mixtures

were centrifuged ; equal volumes from each mixture were removed and the colour was developed with the Folin-Ciocalteu phenol reagent. The colours were measured with a photoelectric colorimeter and, after correction for blank values, are shown in Fig. 5 as optical density (log 100–log per cent T).

From this curve it will be seen that this enzyme showed peak activity at about pH 3–3·5 together with an increasing activity from about pH 6–9, which would have been much greater with denatured haemoglobin. Fig. 5 also shows the activity, determined in the same way, for samples of crystalline trypsin and

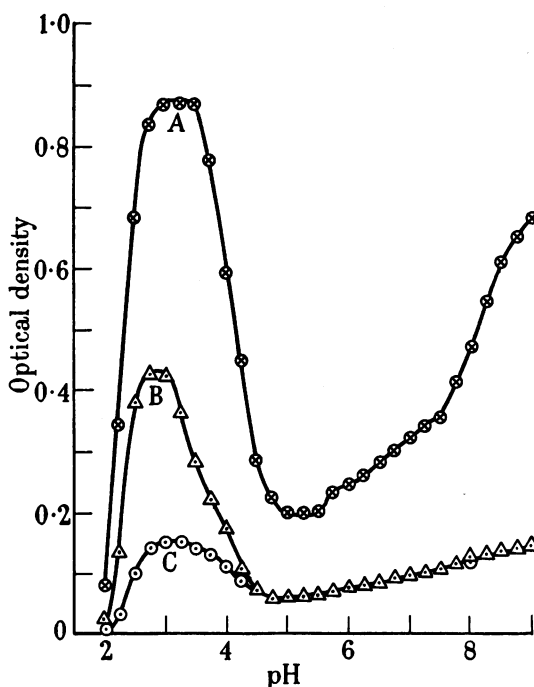

FIG. 5.—The action of an enzyme from sheep pancreas A, crystalline trypsin B, and crystalline chymotrypsin C on crystalline horse haemoglobin at different pH values. Optical density = Density of reading—Density of blank.

From pH 4·75 to pH 9·0 the values for curves B and C were identical except at pH 8 where there was a slight difference as shown in the curve.

crystalline chymotrypsin, both of which were obtained through the kindness of Dr. Taylor. These samples had been crystallized several times as trypsin or chymotrypsinogen, but both show the presence of an enzyme with peak activity at about pH 3. As a result of this finding the crystalline trypsin and chymotrypsin obtained from Dr. Northrop, together with another sample of crystalline trypsin obtained from Dr. E. Work, were examined for their ability to digest undenatured crystalline haemoglobin at pH 3·8. Unfortunately there was insufficient of these materials to do a full test at a series of pH values. However, they all showed rapid digestion of haemoglobin at pH 3·8, and it is possible that on an extended pH range they would have shown results similar to those shown in Fig. 5.

These observations on the enzymes extracted from pancreas suggest that there is present an enzyme with an optimal activity at pH 3–3·5, which can cause both the splitting of the antitoxin molecule and digestion of haemoglobin. The low activity, in terms of antitoxin splitting ability, of crystalline trypsin and chymotrypsin would be explained by the fact that these crystalline enzymes are contaminated with traces of this enzyme active at acid pH values.

Where the enzyme used for antitoxin-splitting was pepsin, it had been assumed that the enzyme responsible was in fact "pepsin" working at a pH value more alkaline than the optimal pH, but in view of the results with pancreatic

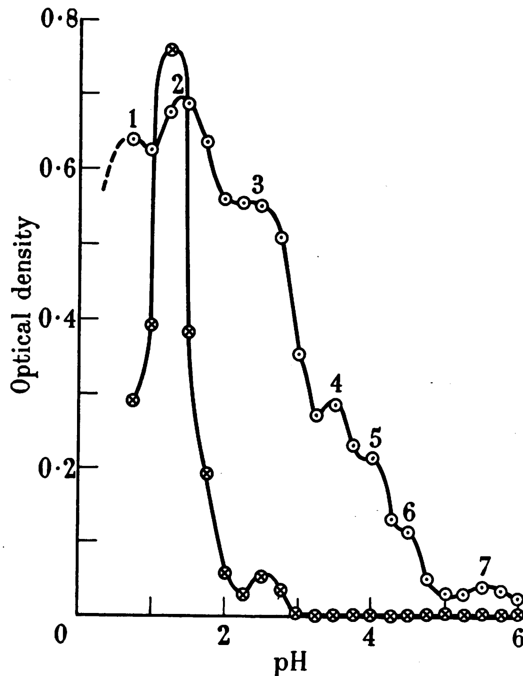

Fig. 6.—The action of pepsin (1/10,000 activity) and a fraction isolated from it on crystalline horse haemoglobin. Optical density = Density of reading − Density of blank. At pH 3·8 the antitoxin molecule is split by the crude pepsin but not by the fraction isolated from it.

extracts it was considered necessary to re-examine pepsin. Starting with pepsin with an activity of 1/10,000 we prepared from it twice crystallized material using the alcohol method of Northrop (1946). The two products were then compared on a weight basis for their ability to digest haemoglobin using a method based on that of Anson (1938). In agreement with Northrop, Kunitz and Herriott (1948) it was found that the crystalline pepsin was five times more active than the starting material. We then assayed these two preparations for their ability to liberate antitoxin from an insoluble substrate using the method described for trypsin. Once again the crystalline pepsin was found to be five times as active on a weight basis as the 1/10,000 pepsin. On this evidence it did not appear that the crystallization process had modified the ratio of activity at pH 1·5 to that at 3·8.

However, it was felt that other methods might throw some light on this problem and we therefore examined the action of pepsin on haemoglobin at a close series of pH values, using the method already described for the pancreatic enzymes. Both the crude 1/10,000 and the crystalline pepsins give curves of the type shown in Fig. 6A, where there is evidence for a series of enzyme-pH peaks (numbered 1–7). Here the pH-activity peaks numbered 4 and 5 might be responsible for splitting the antitoxin molecule, and much work has been done in an attempt to isolate enzymes with activity limited to this pH zone (3·5–4·0). So far we have had little success in this direction, but we have been able to isolate a fraction, by a method to be dealt with in detail elsewhere, with the activity shown in Fig. 6B. This fraction, corresponding to enzyme No. 2 in curve A, is slightly contaminated with enzymes No. 1 and 3, but is inactive at pH values more alkaline than pH 3. It was obviously a matter of interest to test fractions of this type for their ability to split antitoxin at pH 3·8. At the time these fractions were available we had no suitable diphtheria antitoxin substrate, but with the help of Dr. C. L. Oakley the tests were done on *Cl. welchii* antitoxin. These fractions produced virtually no splitting of the antitoxin at pH 3·8, whereas the controls with the original pepsin behaved normally. There appears therefore to be evidence that pepsin contains more than one enzyme ; one of these is concerned with the splitting of antitoxin at pH 3·8.

DISCUSSION.

Evidence has been presented to show that in the case of pepsin, and the enzyme extracted from pancreas, there is an apparent optimal pH at which the enzymes act upon antitoxic protein and split the molecule into two parts, one non-antitoxic and easily denatured, the other carrying the antitoxin and less readily denatured. This explanation of the effect of the enzymes was first suggested by Pope (1938), and subsequently confirmed by Petermann and Pappenheimer (1941). At a temperature of 38° this optimum appears to be at pH 3·8, but at lower temperatures there is evidence that the splitting takes place more rapidly at a pH of 3·2. The apparent variation with temperature is due to the destructive action of acids on antitoxin at more acid pH values at higher temperatures.

Although not dealt with in detail in this paper we have found that papain, rennin, crude maltase, an ultrafiltered concentrate from *Cl. welchii* toxin, etc., all appear to contain an enzyme system optimally active at pH 3·8 and 37°. These are now being investigated in more detail.

An examination of the two major enzymes from pancreas, which are active at alkaline pH values, showed that both crystalline trypsin and chymotrypsin possessed the property of splitting antitoxin at pH 3·8. The amount of enzyme protein required was rather large, and in the two samples examined the chymotrypsin was more active than the trypsin. The sample of chymotrypsin was labelled " 9 × chymotrypsin " and the trypsin " 3 × trypsin," and it is understood that this referred to the number of times the preparation had been crystallized. Our observation that a crude protein fraction isolated from pancreas was very much more active suggested that the crystalline enzymes were slightly contaminated with another enzyme which was responsible for the action on antitoxin at pH 3·8. Further investigation of these enzymes, using crystalline horse haemoglobin as

substrate, at close pH intervals showed that they were active on the acid side with a pH optimum at 3·0–3·5. This activity shown by the crystalline enzymes was the same as that of a fraction isolated from pancreas, assayed in terms of its ability to split antitoxin. From this work it appears that amongst the enzymes extractable from pancreas there is one with the property of splitting antitoxin, and that this is not a property of either trypsin or chymotrypsin except for the trace of enzyme with which they are contaminated. The fact that all samples of crystalline trypsin or chymotrypsin examined have some ability to split antitoxin and digest crystalline haemoglobin at pH 3·8 gives support to the view that the crystallinity of a protein is no guarantee of its purity.

The enzyme pepsin shows a similar apparent optimum at pH 3·8, and our investigations suggest that here again the action is not due to true pepsin, but to another enzyme active at more alkaline pH values. A careful examination of the action of both crude and twice crystallized pepsin at a series of close pH values against crystalline haemoglobin substrate suggests that there may be a series of enzymes present. Unfortunately we have not yet succeeded in isolating material with its activity limited to the necessary pH range, but we have isolated a fraction with its activity sharply limited to pH 1·25 with an absence of activity at pH 3 or higher. This fraction was found to be virtually inactive when tested for its ability to split antitoxin at pH 3·8. In his work on pepsin Buchs (1947) has shown that there is present in pepsin an enzyme of the cathepsin type which shows optimal activity at pH 3·5, and our observations suggest that both pepsin and pancreatic extracts contain enzymes of this type, active at pH 3–3·5, and that these are the ones responsible for the antitoxin splitting. Since all the other enzymes tested show a similar optimal pH value for antitoxin splitting it is probable that they all contain an enzyme of the cathepsin type, but this has not yet been definitely established.* The methods used for fractionation of the enzymes and for determination of the proteolytic activity are not given in detail here as it is proposed to make this the subject of a further communication.

The digestion method, using pepsin, is now a well-established one for the purification of antitoxins for clinical use. Since other crude enzymes have been shown to have the property of splitting the antitoxin molecule, it may be worth while pointing out the advantages of using pepsin. Crude pepsin does not appear to contain any enzymes active at pH values more alkaline than pH 5–6, and the enzyme activity is largely destroyed at slightly alkaline pH values. In the antitoxin refining process the enzyme used is almost completely inactivated after it has done the necessary splitting of the antitoxin molecule. This is not true for either crude trypsin or papain; other enzymes are present which are active at neutrality or slightly alkaline pH values, and these are not readily destroyed or removed from the antitoxin. After the antitoxin molecule has been split at pH 3·8, the portion which carried the antitoxin is considerably more susceptible to the action of the enzymes, which act at more alkaline pH values. By the use of either crude trypsin or papain at pH 3·8 we have been able to purify antitoxin to the same degree as with pepsin, but whereas the pepsin-refined antitoxin shows little or no loss of antitoxin units when kept in the cold room for many years, those prepared using trypsin or papain show very rapid deterioration and fall of antitoxic titre.

* Since this paper was written the presence of a large concentration of an enzyme with this pH optimum has been demonstrated in papain.

SUMMARY.

It has been shown that at 37° the apparent optimal pH value for the action of pepsin in splitting the antitoxin molecule is 3·8 ; at lower temperatures the pH value is nearer 3·2.

A similar optimal pH value has been found for all the other enzymes examined. We have extracted from pancreas and partially purified an enzyme with optimal activity in this region. Samples of crystalline trypsin and chymotrypsin were found to have weak activity at pH 3·8, but this is considered to be due to contamination of these crystalline enzymes with traces of another enzyme.

It is suggested that the enzyme responsible for splitting the antitoxin belongs to the class known as " cathepsin enzymes," and is present in all the crude enzymes examined.

A more detailed examination of pepsin has shown that it also contains a group of enzymes with their pH optima at different values ; one of these, of the cathepsin type, appears to be responsible for antitoxin splitting at pH 3·8. By fractionation of pepsin we have obtained material which shows a sharp optimum activity at pH 1·25 and none at pH 3 (haemoglobin substrate). This fraction had no antitoxin splitting activity at pH 3·8.

All the evidence which we have obtained suggests that a special enzyme of the cathepsin type is necessary for splitting the antitoxin molecule into two parts, one with antitoxin activity and the other without activity.

One of us (C. G. P.) wishes to thank Dr. Northrop, Dr. Taylor and Dr. E. Work for samples of crystalline trypsin or chymotrypsin used in this work.

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