3. Results obtained suggest that the formation of insoluble aggregates from soluble toxin-antitoxin complexes in the equivalence zone may not always be quantitative.

4. Impurities are adsorbed on the α -toxin-antitoxin floccules. The implications of this finding are discussed.

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The Biochemistry of the Gas Gangrene Toxins

5. THE κ -TOXIN (COLLAGENASE) OF *CLOSTRIDIUM WELCHII*

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Maschmann (1937) described an enzyme in young culture filtrates of *Clostridium welchii* (*B. perfringens*) which 'could attack gelatin extensively, but not ovalbumin, casein and fibrin'; he gave details for its partial purification (Maschmann, 1938*a*) and suggested the name 'collagenase' for it (Maschmann, 1938*b*), but later (Maschmann, 1938*c*) withdrew the suggestion without giving reasons.

The possible importance of a collagenase in gas gangrene was discussed by Maschmann (1937, 1938*a, b*) and the subject has been reopened recently by Macfarlane & MacLennan (1945). They observed in cases of clinical gas gangrene in man, and in rabbit muscle injected *in vivo* with *Cl. welchii* culture filtrates, that the trypsin-insoluble collagen framework of the muscle was disintegrated leaving the muscle fibres intact. Teased collagen from human tendon was lysed by *Cl. welchii* culture filtrate; these findings were confirmed by Robb-Smith (1945).

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Macfarlane & MacLennan suggested that *Cl. welchii* collagenase might be responsible for the liberation from the tissues of the host of a non-specific toxic substance(s) which might play an important part in gas gangrene toxæmia. Evans (1945) found that the anti-collagenase titre of *Cl. welchii* type A antisera was not correlated with the protection of guinea pigs against experimentally produced gas gangrene by the sera. At the suggestion of Drs Macfarlane and MacLennan we undertook the work to be described in this paper.

Oakley, Warrack & van Heyningen (1946) showed by titration with a large number of *Cl. welchii* antisera that the muscle-disintegrating substance present in *Cl. welchii* type A filtrates was distinct from α -toxin, θ -toxin and hyaluronidase, and that it was antigenically indistinguishable from the substance in those filtrates which attacks collagen in the form of collagen 'paper', hide powder and 'azocoll'. They proposed that the antigen concerned should be called κ -toxin.

We have succeeded in obtaining preparations of this collagenase free from other toxins of *Cl. welchii* and in a highly purified condition. In addition to its possible clinical interest, this collagenase is remarkable for its high substrate specificity, since it appears to attack only collagen and gelatin.

METHODS

Estimation of collagenase

The antibody-combining power, or antigen content, of collagenase preparations (expressed as κ units/ml.) was measured by the method of Oakley *et al.* (1946).

The enzymic activity (expressed as Q units/ml.) was determined by measuring the amount of dye liberated by the enzyme, under standard conditions, from hide powder to which R acid has been coupled ('azocoll', see Oakley *et al.* 1946). The Q unit is defined as that amount of enzyme which, under the conditions outlined below, will liberate twice as much dye from azocoll as is liberated in a blank.

Preliminary experiments with a photoelectric absorptiometer showed that the amount of dye liberated when a given amount of azocoll was shaken in 10 ml. buffer solution was sufficiently constant to be used as a colorimetric standard. The standard conditions chosen were as follows: 75 mg. of a given batch of 120-mesh azocoll were shaken for 1 hr. at 37° with 10 ml. buffer (equal vol. of M/15 phosphate buffer pH 7.4 and 1% NaCl). The reaction was stopped by filtering off undissolved azocoll. The amount of dye liberated, when enzyme solutions were incubated under the same conditions, was found to be a linear function of the enzyme concentration within the range 0.1–0.8 Q unit/ml., i.e. between the colorimetric ratios standard/unknown of 2–9, where the standard colour was that obtained in the blank as described above.

Since the amount of dye liberated varied with different batches of azocoll, it was important to use large batches. Most of our determinations were carried out with one batch of azocoll, and when a new batch was used it was necessary to determine its characteristics with a collagenase preparation the value of which had already been determined with the first batch. We cannot recommend the method since it cannot be truly standardized, and therefore we have not given it in detail. In our hands it was a rapid and fairly reliable means of following the progress of purification, which we regarded as an expedient to be discarded if a synthetic substrate were found.

Lecithinase (α -toxin) antigen was measured in Le units as described by van Heyningen (1948).

The efficiency of a procedure designed to separate collagenase from lecithinase was expressed by the ratio: Q units of collagenase/Le units of lecithinase in the product, to Q units/Le units in the original material.

Method of estimation of digestion of hide powder. Experiments were made with 120-mesh hide powder (specially prepared by Dr A. C. McDougall from hide powder supplied by Baird and Tatlock Ltd.) which had been washed with water and acetone and dried at 37°. Unless otherwise stated, the hide powder was incubated at 37° for 1 hr. in a total volume of 10 ml., in 25 ml. bottles which were agitated throughout that time. After incubation the mixtures were rapidly cooled to room temperature (within 0.5 min.) and filtered through Whatman no. 41 filter papers.

Controls were run with corresponding heat-inactivated enzyme. Total N determinations were carried out on the filtrates. Results are usually shown as the difference between the total N of the filtrates when active and inactivated enzyme are used.

The action of the enzyme on gelatin. This was followed by determining the total N of the filtrate after the addition to the reaction mixture of 1/9th its volume of 5% (w/v) phosphotungstic acid (A.R.), in $N-H_2SO_4$.

Standard conditions obtained when 40 ml. of 0.5% (w/v) gelatin which had been incubated for 1 hr. at 37° with 5 ml. of enzyme solution were treated with 5 ml. precipitant. In experiments where gelatin solutions stronger than 0.5% were used, they were diluted at the end of the incubation, before adding the precipitant, so that the final concentrations were the same as for 0.5% gelatin. After thorough shaking, and standing for 5 min. the precipitates were filtered off on Whatman no. 3 papers. Heat-inactivated enzyme controls were treated in the same way. Results are shown as the difference between the total N of the filtrates when active and heat-inactivated enzyme preparations are used.

Other estimations. θ -Toxin by the method described by van Heyningen (1948); total nitrogen by micro-Kjeldahl procedure; NH_3 -N by distillation in a Markham still (Markham, 1942) with 10 ml. of the phosphate buffer recommended by Sen (1942); 'tyrosine' by the method of Anson (1938); pH values were measured electrometrically with a glass electrode.

EXPERIMENTAL AND RESULTS

Purification of collagenase

The medium used in most of the work was a nutrient broth (4–5 g. total N/l.) containing 1% peptone, cooked meat and chalk. It was chosen, in preference to the medium described by van Heyningen (1948), because the resultant crude filtrate contains little if any θ -toxin. The organism was *Cl. welchii* type A, strain S 107.

Stage A. The culture filtrate was harvested after 5 hr. growth at 37°, the pH adjusted to 6.8, and $(NH_4)_2SO_4$ stirred in using 60 kg. $(NH_4)_2SO_4$ /100 l. culture filtrate. After standing overnight the scum which floated on the surface was collected and dissolved in water. This solution will be referred to as the starting material.

The further stages in the purification were as follows:

Stage B. Adsorption on calcium phosphate followed by elution with 10% (w/v) $(NH_4)_2SO_4$.

Stage C. Charcoal treatment.

Stage D. Fractional precipitation with $(NH_4)_2SO_4$.

An outline of the procedure finally adopted may now be given.

Starting material from stage A was diluted to a collagenase concentration of 12–15 Q units/ml. and $(NH_4)_2SO_4$ added to a final concentration of 3.75% (w/v). A calcium phosphate precipitate was formed in the solution by adding 80 ml. M- $CaCl_2$ and 200 ml.

0.5M-phosphate buffer (pH 6.8) to each litre of diluted starting material. These additions were made in small portions (using volumes in proportion to total) with vigorous mechanical stirring. After 30–45 min. the precipitate was filtered off by suction on a Buchner funnel, washed with water (without loss of enzyme), and eluted by grinding thoroughly in a mortar with 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$, 250 ml. being used for the precipitate from every litre of

slowly with stirring. After standing overnight at 4° the flocculent precipitate was collected by filtration, with the aid of a little 'Hyflo Supercel' (Johns-Manville Ltd.). The solid on the filter was extracted several times with small volumes of borate or distilled water. The extracts were usually dialyzed against several changes of borate buffer (pH 7.4) in which the enzyme is fairly stable. A typical preparation is shown in Table 1.

Table 1. *Stages and yields in the purification of collagenase*

Stage	Description	Volume (l.)	Q* units/ ml.	κ * units/ ml.	Le* units/ ml.	Stage yield of colla- genase (%)		Overall yield of colla- genase (%)		Non- dialy- zable nitrogen (NDN) (mg./ ml.)	κ units/ mg. NDN	κ /Le
						Calc. from Q-con- tent	Calc. from κ -con- tent	Calc. from Q-con- tent	Calc. from κ -con- tent			
A	Crude filtrate	180	2.1	3.2	3.4	—	—	—	—	0.283	11.3	0.94
	Pptd. with $(\text{NH}_4)_2\text{SO}_4$ (60 kg./100 l.). Scum dissolved in H_2O	21	11.6	29	22.8	64	106	64	106	1.19	24.4	1.27
B and C	First calcium phosphate treatment, giving Eluate I	7.87	23.1	44.6	5.8	75	57	48	61	0.12	374	7.7
	Treatment with charcoal (1.5% w/v)	7.87	19.5	39.2	3.8	85	88	41	54	0.051	770	10.3
	Second calcium phos- phate treatment, giving Eluate II	3.9	23.3	62.5	—	59	79	24	42	0.046	1360	—
	Treatment with char- coal (0.8%)	3.78	17.7	38.5	—	74	60	18	25	0.035	1100	—
	Dialyzed overnight	5.08	12.8	29	< $\frac{1}{2}$	97	101	17	26	—	—	—
D	Fractional precipita- tion with $(\text{NH}_4)_2\text{SO}_4$. Solution of ppt. dialy- zed against borate buffer (pH 7.4)	0.084	604	1230	5.5	78	70	13	18	0.590	2100	224

* Q = collagenase enzyme units; κ = collagenase antigen units; Le = lecithinase antigen units.

diluted starting material. The precipitate was left with the eluent for several hours or overnight at 4°, before filtering. The precipitate was usually eluted again with half the previous volume of 10% $(\text{NH}_4)_2\text{SO}_4$ and the two eluates combined (Eluate I).

Eluate I was stirred for 3–5 min. with an amount of Farnell's L.S. charcoal found to be suitable in a trial experiment (usually about 1%), and filtered. The filtrate was then dialyzed overnight against running tap water. Sufficient solid $(\text{NH}_4)_2\text{SO}_4$ was then added to the dialysate to bring the concentration to 3.75% (w/v) and the adsorption and elution with calcium phosphate repeated as before, to give Eluate II.

Eluate II was then dialyzed against running tap water (if it had retained too much colour it was first subjected to a second charcoal treatment as before). The dialysate was cooled in an ice-water bath and 2 vol. ice-cold 51% (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution added

Determination of optimum conditions for the various stages of the process

(i) Adsorption on calcium phosphate (stage B)

Adsorption of collagenase on calcium phosphate provided the best means of separating it from lecithinase, but the conditions which give the maximum separation consistent with good yields of collagenase are dependent on several factors as outlined below.

Formation of the calcium phosphate precipitate. The precipitate was formed *in situ*, as it was found that not only did aged calcium phosphate give much poorer yields of collagenase, but the separation from lecithinase was also much less efficient.

pH of the buffer used in the precipitation. 50 ml. starting material (28 Q units/ml.; 40 Le units/ml.; 3.2% $(\text{NH}_4)_2\text{SO}_4$) at pH 6.3, were treated with 4 ml. M- CaCl_2 and 10 ml. 0.5M-phosphate buffer at a range of pH values (see Fig. 1). At

Fig. 1. The variation of yield of enzyme, and of efficiency of separation of collagenase from lecithinase, with the pH of the phosphate buffer used in the precipitation of calcium phosphate. The efficiency of separation of collagenase from lecithinase is measured by the separation ratio, i.e. the ratio Q units/Le units in the product to Q units/Le units in the starting material. Yield of collagenase enzyme in eluates x—x. Yield of lecithinase antigen in eluates o—o.

Fig. 2. The variation in yield of enzyme, and efficiency of separation of collagenase from lecithinase, with the concentration of ammonium sulphate in the starting material in the first calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

pH 5.4 and below, no precipitate was formed. The precipitates were eluted with 50 ml. portions of 10% $(\text{NH}_4)_2\text{SO}_4$, and the eluates tested for collagenase and lecithinase content. Fig. 1 shows that the optimum pH appears to be 6.5–7.0, and in all further work 0.5 M-phosphate buffer at pH 6.8 was used. Variation of the pH of the starting material from pH 6.3 to 6.8 did not affect the result.

The concentration of $(\text{NH}_4)_2\text{SO}_4$ in the starting material. To 20 ml. portions of the enzyme preparations (a) or (b) (see below) were added varying amounts of $(\text{NH}_4)_2\text{SO}_4$ and water to 25 ml., 2 ml. M- CaCl_2 and 5 ml. 0.5 M phosphate (pH 6.8). Precipitates were eluted with 20 ml. 10% $(\text{NH}_4)_2\text{SO}_4$ and the eluates tested. Two preparations are described. Enzyme preparation (a): diluted starting material (11.5 Q units/ml.; 23.3 Le units/ml.; Q/Le = 0.49); see Fig. 2. Enzyme preparation (b): dialyzed eluate from a calcium phosphate treatment (Eluate I; 79 Q units/ml., 27 Le units/ml., Q/Le = 2.9); see Fig. 3.

Fig. 3. The variation of yield of enzyme, and efficiency of separation of collagenase from lecithinase, with the concentration of ammonium sulphate in the starting material in the second calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

The curves show that although the details differ for the two preparations, the optimum conditions are similar, and in all further work the concentration of $(\text{NH}_4)_2\text{SO}_4$ was adjusted to 3.75% (w/v) before the calcium phosphate treatment.

The concentration of enzyme in the starting material. Varying amounts of the enzyme preparations (c), (d) and (e) (see below) were diluted to the same volume, the concentration of $(\text{NH}_4)_2\text{SO}_4$ adjusted to 3.75% (w/v), and a calcium phosphate adsorption and elution carried out as usual. Enzyme preparation (c) was starting material derived from van Heyningen's medium (van Heyningen, 1948) (72 Q units/ml., 47 Le units/ml., Q/Le = 1.5) (see Fig. 4). Enzyme preparation (d) was starting material derived from meat-

broth medium (50 Q units/ml., 178 κ units/ml., 120 Le units/ml., Q/Le = 0.42) (see Fig. 5). Enzyme preparation (e) was a dialyzed eluate from a first calcium phosphate treatment (Eluate I) (75 Q units/ml., 250 κ units/ml., 22 Le units/ml., Q/Le = 3.4) (see Fig. 6).

The curves in Figs. 4 and 5 show that, for the first treatment with calcium phosphate, the recovery of collagenase and separation from lecithinase were both improved as the initial concentration of enzyme was decreased, and that

Fig. 4. The variation of yield of enzyme, and separation of collagenase from lecithinase, with the concentration of collagenase in starting material derived from van Heyningen's medium in the first calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

despite differences in the starting materials of preparations (c) and (d) (see Q/Le ratio), the results were qualitatively similar. In order to minimize the inconvenience of working with large volumes, we compromised by diluting the starting material to a collagenase concentration of 12–15 Q units/ml. in all our subsequent routine preparations.

Fig. 6 shows that for the second treatment with calcium phosphate best yields and separation are again associated with low initial enzyme concentrations, but this is much less marked than for the first calcium phosphate treatment. In practice second calcium phosphate treatments were always carried out without dilution.

(ii) Charcoal treatment (stage C)

Much pigment is removed in the calcium phosphate treatments, but sufficient remains to be troublesome in the final concentrates. Farnell's L.S. charcoal will remove most of the pigment and allow twofold purification of collagenase with 80–100% yield. The charcoal treatment also has the advantage that lecithinase is adsorbed to a relatively greater extent than collagenase.

The eluate (containing 10% $(\text{NH}_4)_2\text{SO}_4$) from either a first or second calcium phosphate treatment is stirred for 3–5 min. with an appropriate amount of charcoal as determined by previous experiment (usually about 1%), and then filtered.

Fig. 5. The variation of yield of enzyme, and separation of collagenase from lecithinase, in starting material derived from meat broth medium in the first calcium phosphate treatment. Separation ratio values have the same significance as in Fig. 1. Yield of collagenase enzyme x—x. Yield of collagenase antigen ●—●. Yield of lecithinase antigen o—o.

Adsorption on hide powder

The enzyme can be adsorbed on hide powder and eluted from it, as shown by the following example:

75 g. of 60-mesh hide powder were added to 8.5 l. of dialyzed starting material (containing 49 Q units/ml.; 48 Le units/ml.), stirred for 1 hr., filtered, washed with 1 l. of water and eluted twice by stirring for 1 hr. with 500 ml. of buffer (M/30-phosphate, pH 7.4, containing 0.5% NaCl (w/v)). The eluates were collected by filtration, the hide powder returned to the original supernatant and the process repeated twice. The combined eluates (2.9 l., containing 43 Q units/ml., and 0.5–1 Le unit/ml.) contained the collagenase in 34% yield, and less than 1% of the original lecithinase.

The use of hide powder for routine purification was, however, abandoned because of difficulties of filtration on a large scale, the partial solution of hide powder and its cost.

Fig. 6. The variation of yield of enzyme, and separation of collagenase from lecithinase, with the initial concentration of collagenase in a second treatment with calcium phosphate. Separation ratio values and graphical symbols have the same significance as in Fig. 5.

Properties of the purified enzyme

The purest preparation so far obtained (excluding material which has been subject to dialysis at pH 10.2—see below) contained 2600 Q units of collagenase/ml., 5800 κ -combining units of collagenase/ml., 33 Le units of lecithinase/ml., > 10 units of θ -toxin/ml., 2.27 mg. N/ml., i.e. κ units/mg. N = 2550. (Preparations containing 12,000 κ units/mg. N have been obtained after dialysis at pH 10.2.) The hyaluronidase activity of the preparation has not been tested. The enzyme acts optimally over a range of pH 6.0–7.5, is stable in borate buffers at alkaline and neutral pH, but is relatively unstable at acid pH. (A preparation kept at pH 4.0 for 4 hr. at 4° and then restored to pH 6.8 had lost nearly 40% of its activity.) Pure preparations are not stable in phosphate buffers; e.g. equal volumes of a given preparation were dialyzed for 3 days at 4° against several changes of (a) Palitzsch borate buffer pH 8.0 (Clark, 1928) and (b) M/15-phosphate buffer pH 8.0 with respectively 90 and 47% recovery of collagenase activity.

Dialysis of purified collagenase preparations

against several changes of borate- Na_2CO_3 buffer pH 10.2–10.4 (Clark, 1928) resulted in a considerable reduction in the test dose against κ -antitoxin in azocoll tests, i.e. an increase in the κ -combining power units/ml., sometimes, but not always, accompanied by a corresponding increase in enzymic activity, i.e. rise in Q units/ml.

For example a preparation B 269H (2320 Q units of collagenase/ml., 7140 κ -combining power units of collagenase/ml.) in equilibrium with borate buffer pH 7.4, was redialyzed to equilibrium against borate- Na_2CO_3 buffer pH 10.4; the dialysate was unchanged in volume and contained 4100 Q units of collagenase/ml., and 14,300 κ -combining power units of collagenase/ml., corresponding to an increase of about 75% in the enzymic activity and 100% in the combining power.

It is possible that the dialysis at pH 10.2–10.4 causes the formation of active enzyme from some inactive precursor which also has different immunological specificity.

Parallel electrophoretic investigations by Mr P. A. Charlwood are discussed in the addendum to this paper.

Purified enzyme preparations containing c. 20 Q units of collagenase/ml. are inactivated by heating at 80° for 5 min.

We have confirmed the observation of Maschmann (1938a) that collagenase is partially inactivated in 0.001M-solutions of copper and mercury ions (the latter being most effective). Manganese, cobalt, and iron salts (after long contact with the enzyme) are all inhibitory to varying extents. The decrease in enzymic activity is not accompanied by any change in the combining power. Magnesium and zinc ions appear to be without effect on the enzymic activity.

Kinetic studies

pH optimum. Hide powder (850 mg.) was incubated 1 hr. at 37° with 10 ml. of a mixture of 10 ml. buffer and 2 ml. enzyme, using (a) veronal-acetate-HCl buffer (Michaelis, 1931); (b) M/15-phosphate buffer; (c) Palitzsch borate buffer (Clark, 1928). Determinations of total N were made on the filtrates. Very little variation in the extent of digestion of the hide powder was observed over the range pH 6.0–7.5 (in the case of borate buffer, the lowest pH used was 6.9). Similar results were obtained with gelatin.

Substrate concentration. Column D of Table 2 shows that at concentrations of hide powder higher than 750 mg./10 ml. there is very little further rise in filtrate nitrogen, and further work on the kinetics was done with 850 mg./10 ml. owing to the difficulty in working with higher concentrations. It can be seen from column E (Table 2) that the amount of hide powder dissolved in the controls (heat inactivated enzyme) is approximately a constant percentage of the total amount present over the range 250–1000 mg./10 ml.

Table 2. *The optimum concentration of substrate for the action of collagenase on hide powder*

(Final collagenase concentration 0.28 Q units/ml. Buffer: Palitzsch borate pH 7.4.)

Hide powder concentration (mg./10 ml.)	Nitrogen content of filtrate (mg. N/10 ml.)		Nitrogen content of filtrate due to enzyme action (mg. N/10 ml.) (B - C)	Hide powder dissolved in controls* (%) $\left(\frac{C}{A} \times \frac{10^4}{15.4}\right)$
	When active enzyme used	When heat-inactivated enzyme used		
A	B	C	D	E
100	8.4	3.7	4.7	24.0
250	17.0	7.6	9.4	19.8
500	28.8	14.0	14.8	18.2
750	40.3	20.6	19.7	17.9
1000	49.4	29.4	20.0	19.1

* In a separate experiment it was found that the hide powder used contained 15.4% nitrogen.

Table 3. *The optimum concentration of substrate for the digestion of gelatin by hide powder*

N (mg./ml.) in gelatin substrate solution	Mixture incubated			Nitrogen content of filtrate (mg. N/100 ml.)			Col. G recalculated to refer to 80 ml. gelatin (mg. N)
	Gelatin (ml.)	Buffer* (ml.)	Enzyme† (ml.)	When active enzyme used	When heat- inactivated enzyme used	Due to enzyme action (E - F)	
A	B	C	D	E	F	G	H
5.57	10	1.0	0.25	3.27	0.37	2.90	23.2
2.73	20	2.0	0.50	5.55	0.29	5.26	21.0
1.37	40	4.0	1.00	7.09	0.42	6.67	13.3
0.69	80	8.0	2.00	8.50	0.53	7.97	8.0

* Palitzsch borate pH 7.2.

† Active enzyme contained 21 Q units of collagenase/ml.

In determining the substrate concentration optimum for gelatin, the mixtures, made up as shown in Table 3, were incubated 1 hr. at 37°. Then sufficient ice-cold buffer (Palitzsch borate pH 7.2 in which all the solutions had been prepared) was added to bring the final volume to 90 ml., followed by 10 ml. precipitant (5% (w/v) phosphotungstic acid in $N-H_2SO_4$). Owing to the difficulty of preparing standard solutions of gelatin, the concentrations of the gelatin solutions used are indicated in column A of Table 3 by their nitrogen content. The values of mg. N/100 ml. filtrate due to the enzyme activity are shown in column G, Table 3. Column H gives the calculated values for the total amount of nitrogen 'soluble' under the given conditions (i.e. when 1 vol. of precipitant is added to 9 vol. of a mixture containing c. 0.6 mg. N/ml.) which would be produced by 2 ml. of the enzyme solution used, acting on 80 ml. of gelatin of the concentration given in column A in a total volume of 90 ml. A final gelatin concentration during incubation corresponding to about 5 mg. gelatin N/ml. seems to be about optimal.

Activity curves. Hide powder: see Fig. 7, curve A. M/15-phosphate buffer (pH 6.5), 850 mg. hide powder/10 ml., and incubation for 1 hr. at 37° were used.

Fig. 7. Collagenase activity curves. A, with hide powder; B, with gelatin as substrates.

Gelatin: see Fig. 7, curve B. 10 ml. amounts of a solution of gelatin (5 mg. N/ml.) in borate buffer (pH 7.2) were incubated with amounts of enzyme

as shown (total vol. during incubation, 11.25 ml.). After 1 hr. at 37°, 78.75 ml. ice-cold buffer was added, followed by 10 ml. precipitant; remainder of the procedure as usual. Controls: heat-inactivated enzyme treated in exactly the same way.

Interpretation of activity curves. Bergmann (1933) found that the enzymic digestion of gelatin sheets, the area of which remained constant during digestion, could be described by the formula: $x = kt\sqrt{E}$, where x = percentage digestion; k = constant; t = time; E = percentage enzyme concentration, and that the formula also held for the early part of the digestion, of fibrous collagen, i.e. these reactions followed the Schutz rule. He suggested that this could be explained by the assumption that the digestion of collagen fibres proceeds only from their ends. Fig. 8

Fig. 8. Data from activity curves replotted to show variation in activity with the square root of the enzyme concentration. A, hide powder; B, gelatin.

shows that the digestion of hide powder and gelatin (in solution) by low concentrations of *Cl. welchii* collagenase in a given time also proceeds approximately according to a similar expression, $x = k\sqrt{E} - a$, where a is a constant, possibly due to partial inactivation of the enzyme during the course of the incubation, and that the kinetics of digestion of undissolved hide powder and dissolved gelatin are similar. Therefore we feel that Bergmann's suggestion that collagen fibres are digested only from the ends needs re-examination.

Substrate specificity

Proteins other than collagen or gelatin. 5 ml. of substrate solution or suspension (containing about 2% protein) were incubated with 1 ml. enzyme

solution for 1 hr. at 37°, then 10 ml. 0.3M-trichloroacetic acid were added, and the solution filtered through Whatman no. 3 filter papers. Controls: 5 ml. substrate, 10 ml. 0.3M-trichloroacetic acid added, then 1 ml. enzyme, filtered. The extent of digestion was estimated by either 'tyrosine' or total N determination on filtrates.

(a) Urea-denatured haemoglobin, prepared according to Anson (1938): little or no effect in the absence of cysteine, slight digestion in the presence of cysteine.

(b) Casein (British Drug Houses Ltd. L.W.S.): little or no effect.

(c) Normal horse serum: no effect.

(d) Urea-denatured normal horse serum: little or no effect.

(e) Heat-denatured horse serum albumin: no effect in the absence of cysteine; in presence of cysteine, about 10% of the substrate was digested. The experiment was repeated with a more highly purified preparation of collagenase, which showed no activity against heat-denatured horse serum albumin, even in the presence of cysteine and during 20 hr. incubation at 37°.

(f) Clupein sulphate: no action even during 20 hr. in the presence of cysteine.

The final collagenase concentration in experiments (a)-(d) was 16 Q units/ml., and in (e) was 7 Q units/ml. For comparison, it may be calculated from the data in Table 2, that collagenase in a final concentration of 0.28 Q units/ml. digests nearly 25% of the hide powder present (in 2.5% suspension) in 1 hr. at 37°.

Titration of groups liberated by the action of collagenase on gelatin. After incubation of gelatin with collagenase samples were titrated as follows:

(1) For amino nitrogen by the method of Pope & Stevens (1939).

(2) By the KOH-ethanol method (Grassmann & Heyde, 1929).

(3) By the HCl-acetone method (Linderstrøm-Lang, 1928).

In the control experiment a corresponding amount of heat-inactivated enzyme was used, the values were subtracted from those obtained where the active enzyme was used, and the result expressed in terms of m.equiv. of $-\text{NH}_2$ or $-\text{COOH}$ formed by the enzyme in the whole mixture incubated (50 ml.). The conditions of the experiment were such that method (2) should measure the total acids formed, and method (3) the total $-\text{NH}_2$ groups formed. (For a critical study of these methods, see Richardson, 1934.) Method (1) provides a check on the others. The experiment was carried out on duplicate mixtures. The results (Table 4) were consistent with the splitting of a peptide bond.

Possible synthetic substrates. The following substances were found to be unaffected by collagenase: hippuramide, L-glycylproline, L-carbobenzylglycylglycine, pyroglutamic acid, O-acetyl-L-hydroxyproline, ON-diacetyl-L-hydroxyproline.

Table 4. The $-\text{NH}_2$ and $-\text{COOH}$ groups liberated by the action of collagenase on gelatin

(50 ml. gelatin solution [final concentration of gelatin N, 5.6 g./l.] with sodium chloride, 1% (w/v) incubated at 37° for 20 hr. with collagenase (1 Q unit/ml.) or the corresponding amount of heat-inactivated enzyme. All solutions adjusted to pH 7.0 before mixing. Toluene (2 drops) added to each mixture.)

Method of estimation	m.-equiv. of $-\text{NH}_2$ or $-\text{COOH}$ formed
Pope & Stevens's (1939) copper method	1.90 ($-\text{NH}_2$)
HCl-acetone titration	2.08 ($-\text{NH}_2$)
KOH-ethanol titration	2.14 ($-\text{COOH}$)

Neutralization by antibody

It was shown by Oakley *et al.* (1946) that antisera produced in horses in response to immunization with culture filtrates of *Cl. welchii* type A generally contained antibody which inhibited the action of collagenase on collagen in the form of azocoll and collagen 'paper'. It was therefore to be expected that such antisera would neutralize the gelatin-splitting activity of our enzyme preparations. However, not only could inhibition not be demonstrated satisfactorily, but in some cases mixtures containing excess antiserum were apparently more active than when no antiserum was present.

Example: Varying amounts of antiserum R 8537 (from the same batch as the standard serum R 8531 of Oakley *et al.* (1946) and defined as containing 180 anti- κ units/ml.) were added to 1.0 ml. enzyme preparation (26 Q units/ml., 102 κ -combining units/ml.) diluted in sufficient borate buffer (pH 7.4) to make the final volume 7.5 ml. After standing 0.5 hr., 5 ml. amounts of the mixtures were incubated with 40 ml. gelatin solution (0.73 mg. N/ml.) for 1 hr. at 37° . 5 ml. of the usual precipitant were added and total N determinations were carried out on the filtrates. The controls consisted of a parallel series of mixtures, using heat-inactivated enzyme, which were incubated in exactly the same way.

The results are given in Table 5. All the mixtures were tested for activity against azocoll by the in-

cubation overnight at 37° of 0.5 ml. of mixture with 1 ml. 'azocoll-manuocol' suspension (Oakley *et al.* 1946). The results are shown in Table 5, column F. As expected, only the first mixture gave a strongly positive reaction (+ +), while all the others were completely negative. Mixtures 2 and 3 were undoubtedly over neutralized as far as activity against azocoll is concerned. The failure to neutralize the activity against gelatin might be due to one of several causes:

(a) Part, if not all, of the 'gelatinase' activity might be due to an immunologically distinct enzyme. If this were so, the 'gelatinase' would appear to be a very poor antigen, since several antisera having very varied contents of other antibodies have been shown to be ineffective in neutralizing this activity against gelatin.

(b) Dissociation of the complex formed by the enzyme with the homologous antibody. This appears an unlikely explanation in view of the fact that the final concentration of antiserum was over 50 anti- κ units/ml. and the incubation time only 1 hr., while the usual concentration in testing for combining power (κ -unitage) is less than 1 anti- κ unit/ml. and the time of incubation overnight.

(c) The anti-collagenase antibody may prevent gross particles of hide powder, but not the comparatively small molecules of gelatin, from reaching the catalytic centre of the enzyme. It has previously been observed that in some cases enzyme-anti-enzyme complexes are formed which retain all the activity of the uncombined enzyme, for example, the work of Campbell & Fourn (1939) on catalase.

None of the above explanations accounts for the rise in activity which has sometimes been observed in the presence of serum. That this is not due to any direct action of the serum on gelatin is shown by the controls (5 and 6, Table 5); the rise shown (over control 4) is probably due only to non-precipitable nitrogenous material in the added serum, and is in any case so small as to be negligible in the present discussion.

Table 5. The effect of serum containing anti-collagenase antibody on the action of a collagenase preparation on gelatin

Mixture no.	Added to 40 ml. gelatin		Nitrogen content of filtrate (mg. N/50 ml.)	Nitrogen content of filtrate produced by active enzyme (mg. N/50 ml.)	Activity of mixtures against azocoll
	κ units of collagenase	Anti- κ units of serum			
A	B	C	D	E	F
1	68	0	4.64	4.23	+ +
2	68	240	6.96	6.27	No detectable activity
3	68	360	8.29	7.39	"
4	<1/3	0	0.41	—	"
5	<1/3	240	0.69	—	"
6	<1/3	360	0.90	—	"

DISCUSSION

According to the theories of Bergmann and his colleagues (Bergmann & Fruton, 1941) the specificity of proteolytic enzymes is governed by the nature and arrangement of the amino-acid residues on either side of the peptide bond that is hydrolyzed. The arrangement of amino-acids to which a given enzyme is specific may not occur in certain proteins, in which case those proteins would not be attacked by the enzyme; but it is surprising that out of the number of proteins tested the appropriate conditions of specificity for the collagenase of *Cl. welchii* appears to be present only in collagen (and its derivative, gelatin). This protein differs from other proteins in its high content of proline and hydroxyproline, but the narrow specificity of collagenase does not appear to be connected with these amino-acids. The failure of the enzyme to hydrolyze glycylproline or carboxybenzyloxypolyglycine indicates that the enzyme is neither a prolidase (Bergmann & Fruton, 1937) nor a prolinase (Grassmann, Schoenebeck & Auerbach, 1932).

We were prepared to consider the possibility that collagenase is not a peptidase, but that it dissolves collagen and liquefies gelatin as a result of its catalyzing some reaction other than the hydrolysis of a peptide bond. For example, it is conceivable, as Dr A. Neuberger suggested to us, that collagen and gelatin might contain ester linkages between the —OH groups of hydroxyproline residues and those carboxyl groups of glutamic acid that are not involved in peptide bonds. Hydrolysis of such linkages by an esterase might result in the solution of collagen or the liquefaction of gelatin. For this reason we tested the action of our enzyme preparations on *O*-acetyl-L-hydroxyproline and on *ON*-diacetyl-L-hydroxyproline, but no hydrolysis resulted.

On the other hand, the enzyme does appear to catalyze the hydrolysis of peptide bonds since amino-acids are liberated by it from gelatin, as is evident from our results. (Maschmann (1937) estimated his gelatinase by titrating the liberated amino groups.) The production of free amino groups is very slow compared with the solution of collagen or the liquefaction of gelatin.

The increase in κ -combining units of collagenase antigen (i.e. the decrease in the κ test dose), which occurred when partially purified preparations of the enzyme were dialyzed against borate- Na_2CO_3 buffer

(pH 10.2–10.4), was confirmed on several occasions. It was often accompanied by an increase in enzyme activity (as measured by a rise in Q units/ml.). The enhancement of the combining power and enzymic activity was retained on redialyzing to equilibrium against pH 7.4 borate buffer. It is possible that the dialysis at pH 10.2–10.4 causes the formation of collagenase from some inactive precursor, which also has different immunological specificity. The parallel electrophoretic investigations described by Mr P. A. Charlwood in the addendum to this paper lend some support to this view, since a comparison of the electrophoretic properties (at pH 7.4) of a preparation before and after an intermediate dialysis at pH 10.2 (in borate- Na_2CO_3) shows that this treatment has produced some irreversible change in the protein constitution of the mixture.

SUMMARY

1. The preparation, from culture filtrates of *Cl. welchii* type A, of a proteolytic enzyme, 'collagenase' (κ -toxin), which appears to attack only collagen and gelatin, is described. It has been obtained substantially free from the α - and θ -toxins of *Cl. welchii* and purified (with respect to non-dialyzable nitrogen) about 200-fold.
2. The partially purified enzyme is optimally active over the pH range 6.0–7.5, and is stable in solution in borate but not in phosphate buffers.
3. An increase in activity and antibody-combining power after dialysis against borate buffer at pH 10.2 was observed.
4. Sera neutralizing the activity of collagenase-containing filtrates against collagen do not inhibit their activity against gelatin.
5. The electrophoretic properties of partially purified preparations are discussed in an addendum.

We are grateful to Dr R. G. Macfarlane and Dr J. D. MacLennan for suggesting this problem. We also wish to thank Miss G. H. Warrack for many immunological tests, Miss A. G. Kennedy for many nitrogen and other determinations, Mr G. A. Spiby for much technical assistance, Mr J. C. Macsween and the staff of the toxin laboratories for the provision of the crude filtrates, Dr S. Wilkinson of the Wellcome Chemical Research Laboratories for synthesizing the various peptides, etc., mentioned and Dr A. C. McDougall of the Wellcome Chemical Works for the preparation of 120-mesh hide powder. We are grateful to Dr C. L. Oakley for many helpful discussions.

Addendum. Electrophoresis of Collagenase Preparations

By P. A. CHARLWOOD

Experimental. The electrophoresis of collagenase preparations was carried out in a Tiselius apparatus at $+0.5^\circ$, the diagonal schlieren method being employed. Preparations were dialyzed against borate or borate-carbonate buffer at the required pH. The buffer was renewed every 12 hr., and equilibrium was considered to be attained when no difference could be detected between the refractive indices of fresh buffer and buffer which had been in contact with the sample for 12 hr. Potential gradients employed in the U-tube ranged from 2.7 to 3.1 V./cm.

Fig. 9B). Again the residues were collected (B 287A), dialyzed back to pH 7.4 (B 287B) without significant change in volume and Table 6 shows that there was no loss in κ -unitage during the return to pH 7.4. A final run was then carried out on preparation B 287B (see Fig. 9C).

Results of electrophoretic experiments. The electrophoretic pattern of B 285B (Fig. 9A) shows three peaks. The pattern of B 286B (Fig. 9B) also shows three peaks, but is considerably different, as the fastest-moving component now constitutes the

Table 6. *Collagenase preparations used in the electrophoretic experiments*

Sample	Q units/ml.	κ units/ml.	mg. N/ml.	κ units/mg. N	θ units/ml.	Le units/ml.
B 285B	3,200	7,000	3.22	2,180	<1	37.8
(B 286A)	(2,220)	(5,100)	—	—	—	—
B 286B	3,000	11,100	1.79	6,200	—	—
(B 287A)	(2,360)	(7,140)	—	—	—	—
B 287B	2,050	7,500	1.37	5,500	—	—

The collagenase preparation B 285B was dialyzed at pH 7.4 and a run carried out (see Fig. 9A). The material recovered from the U-tube (unavoidably diluted with buffer) was combined with the rest of B 285B, and filtered. The filtrate (B 286A) was dialyzed against buffer at pH 10.2; the dialysate (B 286B) was unchanged in volume and Table 6 shows that this treatment resulted in a large increase (over 200%) in the total κ units present. Preparation B 286B was submitted to electrophoresis (see

major portion of the protein. The final material, B 287B (Fig. 9C) is clearly different from B 285B. These two, being at the same pH, should be almost identical if the sequence of pH changes had not affected the material present. The fastest-moving component in B 287B is present in much greater proportion than in B 285B.

The above series of changes in the curves correlates with the alteration in combining power. It would appear that the combining power is associated

Fig. 9. Electrophoresis of collagenase. Anode (ascending) limb curves are shown. Migration is from left to right.

chiefly or wholly with the fast component. It would be of great interest to separate electrophoretically a pure specimen of this component and to ascertain whether or not this is the case. The chief difficulty lies in achieving a sufficiently good separation of pure material from the amounts of the preparations available.

Mobility determinations were made at pH 10.2 and 7.4. The results are shown in Table 7. They are uncorrected for buffer viscosity, and are also liable to contain errors caused by the inability to maintain a very steady current, owing to large fluctuations of the mains supply.

Table 7. *Mobilities of components present in collagenase preparations, expressed in cm./sec./V./cm. $\times 10^{-5}$*

pH	Component		
	Fast	Middle	Slow
10.2	-7.29	-5.96	-5.41
7.4	-5.25	-4.37	-3.76

The above results have been confirmed qualitatively by runs on other batches of collagenase preparations.

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The Colorimetric Determination of Stilboestrol and Dienoestrol

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A preliminary account has been given (Warren & Goulden, 1945) of investigations that have been carried out on methods proposed for chemical assay of synthetic oestrogens. Two methods were studied. One (Dingemans, 1939) is based upon the development of a red colour by the oestrogen in the presence of antimony pentachloride; the other (Malpress, 1945) relies on the measurement of the yellow colour obtained by nitration of the oestrogen followed by neutralization. The antimony reaction proved to be very sensitive. By its use the instability of dien-oestrol in solution was demonstrated. On the other hand, stilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene) appears to be quite stable in solution in a

variety of solvents. It was subsequently found (Wilder Smith & Williams, 1945) that the oestrogenic activity of solutions of dienostrol shows a similar decrease with time. Hence, chromogenic activity towards antimony pentachloride and oestrogenic activity appear to run parallel. On the other hand, solutions of dien-oestrol that show very low activity in the antimony pentachloride reaction continue to react strongly in Malpress's test.

In the present paper details of these experiments are given, together with the results of some attempts to apply the antimony reaction to the estimation of synthetic oestrogens in urine of subjects treated therapeutically with these substances.