cautious addition of ether. Recrystallization from methanol-ether gave *microneedles*, m.p. 268–270° (decomp.). (Found: N, 13.0%. $C_{37}H_{55}O_7N_7S \cdot HCl$ requires N, 12.6%.)

SUMMARY

1. Two optically isomeric open-chain pentapeptide esters have been synthesized. These peptides have the same sequence of amino-acid residues as gramicidin S.

2. A crystalline acylpentapeptide amide has been synthesized. This compound is structurally analogous to the natural antibiotic in that its constituent

amino-acids are arranged in the same sequence and are of the same optical configuration as those of gramicidin S and that its only reactive group is the δ -NH₂ of the ornithine residue.

3. These and other newly synthesized peptides were tested, *in vitro*, for antibacterial activity.

4. The bearing of the results on the question of the origin of antibiotic activity in gramicidin S is discussed.

We are indebted to Dr A. T. Fuller for the biological tests summarized in Table 1, and to Mr J. Smiles for the photograph of crystalline acylpentapeptide ester.

REFERENCES

- Albertson, N. F. & Archer, S. (1945). *J. Amer. chem. Soc.* **67**, 2043.
 Bach, S. J. (1946). *Nature, Lond.*, **158**, 376.
 Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1947). *Biochem. J.* **41**, 596.
 Cox, G. J. (1928). *J. biol. Chem.* **78**, 475.
 Ehrensvärd, G. C. H. (1947). *Nature, Lond.*, **159**, 500.
 Fischer, E. (1901). *Ber. dtsch. chem. Ges.* **34**, 433.
 Harris, J. I. & Work, T. S. (1950). *Biochem. J.* **46**, 196.
 Hunter, A. (1939). *Biochem. J.* **33**, 27.
 Hunter, A. & Dauphinee, J. A. (1930). *J. biol. Chem.* **85**, 627.
 Karrer, P. & Veer F. C. van der S. (1932). *Helv. chim. Acta*, **15**, 746.
 Neuberger, A. & Sanger, F. (1943). *Biochem. J.* **37**, 515.
 Pedersen, K. O. & Synge, R. L. M. (1948). *Acta chem. Scand.* **2**, 408.
 Prelog, V. & Wieland, P. (1946). *Helv. chim. Acta*, **29**, 1128.
 Rivier, M. H. (1907). *Bull. Soc. chim.* (4) **1**, 733.
 Synge, R. L. M. (1948). *Biochem. J.* **42**, 99.
 Znamenskaya, M. P., Agatov, P. A. & Belozerskiĭ, A. N. (1948). *Doklady Akad. Nauk S.S.S.R.* **59**, 95. Through *Chem. Abstr.* **42**, 6402 (1948).

Proteolytic Enzymes of *Clostridium welchii*

By ETHEL BIDWELL*

The Wellcome Research Laboratories, Beckenham, Kent

(Received 2 December 1949)

Culture filtrates from *Clostridium welchii* Type A contain an enzyme, collagenase (called κ -toxin by Oakley, Warrack & van Heyningen, 1946), which dissolves native collagen, softens muscle and also attacks degraded collagen as in hide powder and 'azocoll' (hide powder coupled with a red dye). The partial purification and some biochemical properties of this enzyme were described by Bidwell & van Heyningen (1948) and by Bidwell (1949*a*); observations on its effect *in vivo* and its action on gelatin were made by Oakley, Warrack & Warren (1948).

Walburn & Reymann (1934) found that filtrates from a strain of the lamb dysentery bacillus (*Cl. welchii* Type B) liquefied gelatin optimally at pH 7. Oakley *et al.* (1948) showed that *Cl. welchii* Type B produced a substance which attacked hide powder, azocoll and gelatin, but appeared to be without effect on undegraded collagen and muscle; it was antigenic and was designated the λ -antigen of *Cl.*

welchii. It was inhibited by a substance in normal horse serum which is not identical with the trypsin inhibitor (Bidwell, 1949*b*). The present paper shows that the λ -antigen of Oakley *et al.* (1948) has the properties of a proteolytic enzyme; it will be referred to as ' λ -enzyme'. Its partial purification and properties will be described and compared with those of collagenase and the 'secondary' enzyme formed by the treatment of collagenase preparations with mild alkali or heat (Bidwell, 1949*a*).

METHODS

Estimation of enzymic activity. The ability of λ -enzyme preparations to disintegrate azocoll was determined in '*R* units' in terms of a standard dried preparation by measuring the amount of dye liberated from azocoll under standard conditions. The standard preparation was crude culture filtrate of *Cl. welchii* Type B grown on meat broth medium containing 1% Parke Davis peptone, which was preserved by drying from the frozen state. The standard preparation contained 0.2 *R* unit of λ -enzyme/mg. by definition. The

* Now at Radcliffe Infirmary, Oxford.

magnitude of the R unit was so chosen as to indicate approximately the same level of activity against azocoll as that of 1 Q unit of collagenase (Bidwell & van Heyningen, 1948).

A standard curve was constructed as follows: 100 mg. amounts of 60-mesh azocoll (Oakley *et al.* 1946) were shaken for 30 min. at 37° with 10 ml. enzyme diluted in phosphate buffer-saline (equal vol. of $M/15$ -phosphate buffer pH 7.4 and 1% (w/v) NaCl). Undissolved azocoll was filtered off through Whatman no. 31 or 41 papers. The filtrate was diluted to a convenient depth of colour (usually 3–6 times) and matched in a visual colorimeter using a green filter (Ilford 604) against a standard colour (1% (w/v) solution of methyl red in 60% (v/v) ethanol as stock solution; diluted for use 1/100 in 0.1 N -HCl). The colorimetric ratio, standard methyl red/standard enzyme preparation, was plotted against the enzyme units used to give an activity curve from which the activity of unknown solutions could be obtained in terms of the standard preparation. Fig. 1 shows the standard activity curve for λ -enzyme; the curve for collagenase under the same conditions is also shown. The two curves are very similar.

Fig. 1. Standard activity curves for the determination of the activity against azocoll of λ -enzyme (in R units) and collagenase (in Q units). Collagenase (Q units): $-\times-\times-$. λ -Enzyme (R units): $-\bullet-\bullet-$. Varying amounts of enzyme in a total volume of 10 ml. phosphate buffer-saline (pH 7.4) were incubated with 100 mg. of azocoll (batch B478C) for 0.5 hr. at 37°. Colours of filtrates matched against 0.01% methyl red in 0.1 N -HCl.

The ability of λ -enzyme preparations to hydrolyse casein and urea-denatured haemoglobin was determined by methods based on that of Anson (1938) for trypsin. Since this method is not applicable to very impure preparations on account of high blanks, a partially purified λ -enzyme preparation (B473E': 57 R units/mg.) was used as subsidiary standard. The substrates were 2.5% (w/v) casein (Glaxo, L.W.S.) in veronal-acetate-HCl buffer (Michaelis, 1931) pH 7.1 and urea-denatured haemoglobin prepared according to Anson (1938). Enzyme (1 ml.) was added to 5 ml. substrate at 37°, mixed, incubated at 37° for exactly 10 min., 10 ml. 0.3 M -trichloroacetic acid added, shaken, and the mixture filtered after 30 min. (Whatman no. 3). In the controls trichloroacetic acid was added to the substrate before the enzyme. The 'tyrosine equivalent' of the filtrates was determined, the 'colour value' calculated and the enzyme unit fixed exactly as described by Anson (1938) as follows:

1.61 g. B473E' = 1 enzyme unit, casein as substrate, [E.U.] casein,

2.56 g. B473E' = 1 enzyme unit, haemoglobin as substrate, [E.U.] Hb.

Determinations of antibody combining power

λ -Antigen combining power values (Oakley *et al.* 1948) were determined by a method analogous to that used for κ -toxin combining power (Oakley *et al.* 1946). The standards fixed by Dr Oakley and Miss Warrack were sera refined by the pepsin process (Pope, 1938; 1939a, b) which destroys the non-specific λ -inhibitor of serum. The serum RX 5417 was defined as containing 8000 anti- λ units/ml.; subsidiary standard RX 7463 contained 1100 anti- λ units/ml.

Each of a series of tubes contained 10 units anti- λ antibody together with amounts of enzyme decreasing by 10% in a total volume of approx. 2 ml. The mixture was allowed to stand 0.5 hr. at room temperature; 1 ml. of azocoll suspension (300 mg. to 100 ml.) was added to each tube. After incubation at 37° for 16–18 hr. the tests were read as described by Oakley *et al.* (1946).

λ -Combining power values using gelatin as substrate were determined by the gelatin-agar tube method of Oakley *et al.* 1948. Each test mixture contained 1 unit of an arbitrary standard serum (RR 7480 $\equiv$ 300 units/ml.).

β -Toxin combining power values were estimated by intracutaneous injection into guinea pigs (Glenny, Llewellyn-Jones & Mason, 1931); ϵ -toxin by the method of Batty & Glenny (1947) using as indicator the appearance of a specific necrotic reaction in rabbits. The antibody units were those in general use in these laboratories.

Other estimations. Total N was determined by the micro-Kjeldahl procedure; pH values were measured electrometrically using a glass electrode. Bacterial growth was estimated in terms of bacterial nitrogen as follows. Samples of the culture were centrifuged, the organisms washed twice with 0.05% (v/v) acetic acid (Mueller, 1935) and total N determinations carried out.

Buffers. Where no reference is given the composition of the buffers used was that given by Clark (1928).

EXPERIMENTAL

Choice of medium. The first crude filtrate examined was from a medium which had been in general use in these laboratories for the growth of *Cl. welchii* Type B, a nutrient broth containing 1% (w/v) Parke Davis peptone and meat and referred to as P.D. medium. This filtrate was highly active in disintegrating azocoll (10 R units of λ -enzyme/ml.), but later filtrates obtained with the same batch of medium were less satisfactory (5 R units/ml. or less). Since this batch of medium had been prepared with the last remnants of our stock of American peptone (irreplaceable under conditions then prevailing), it was essential to find an alternative medium. Many attempts were made to use one of a considerable variety of 'toxin media' available, and also to use media made according to the formula for P.D. medium but with other peptones, including a tryptic digest of meat, substituted for the Parke Davis brand, but all were unsuccessful (<2 R units/ml., often <1 R unit/ml.).

The effect of adding various substances to the medium. The basal medium for these experiments was made according to P.D. formula, but papain-digested horse muscle was used

in place of Parke Davis peptone. The addition of glucose resulted in an increase in growth but there was marked inhibition of λ -enzyme production. The addition of calcium or magnesium to the medium has been stated to promote production of bacterial protease although without effect on growth (Merill & Clark, 1928; Haines, 1931, 1932); there was no improvement in growth of *Cl. welchii* Type B on further addition of Ca or Mg to a basal medium containing initially 33 mg. $\text{Ca}^{++}/\text{l.}$ and 19 mg. $\text{Mg}^{++}/\text{l.}$ The addition of Mg had no effect on λ -enzyme production, but Ca (1–4 ml. $\text{M-CaCl}_2/600$ ml. medium) produced a small but definite rise (increase in R value up to 50 % over control). Since a precipitate (probably Ca phosphate) appeared in the medium on the addition of CaCl_2 , the increase in λ -enzyme might have been due to the removal of inhibitory substances (e.g. trace metals) and not to an increase in Ca ion concentration. Part of a batch of medium was therefore 'deferrated' (i.e. a Ca phosphate precipitate was formed in the medium and removed by filtration), but no improvement resulted. The addition of asparagine (Diehl, 1919) was without effect.

Final medium. A pancreatic digest of casein (Adams & Hendee, 1945) was tried and found to be so successful for the production of λ -enzyme that no further work on meat digests was done. In the medium as finally used values of 10–20 R units of λ -enzyme/ml. were regularly obtained; occasionally even higher values have been found.

The medium was developed by Adams & Hendee (1945) for the production of high value *Cl. welchii* α -toxin. The casein is digested using a relatively large proportion of minced whole pancreas. Adams, Hendee & Pappenheimer (1947) have shown that the pancreas provides a toxigenic factor (for α -toxin production) apart from its function in digesting the casein. Adams & Hendee (1945) recommended insoluble dextrin as an energy source (see also Logan, Tytell, Danielson & Griner, 1945), but as this was not available, the less soluble fraction of a yellow dextrin was used in the preliminary experiments. Adams *et al.* (1947) stated that potato starch may be used instead of insoluble dextrin for the production of α -toxin; it was found to be rather better than the dextrin for λ -enzyme production, and was used in all subsequent work at a final concentration of 0.25 % (w/v). This amount of starch gave about 50 % more λ -enzyme than was obtained in the absence of starch.

It may be remarked that all our attempts to produce high value α -toxin by growing *Cl. welchii* Type A on this medium, for which it was originally designed, have been unsuccessful. Only in one instance was even a very low level of α -toxin attained, although we have used Adams & Hendee's (1945) own strain which they kindly sent to us as well as the classical S107. This may indicate that although we adhered closely to the directions given by Adams & Hendee (1945) the medium as prepared by us differs in some essential point from that of the originators, possibly due to a difference in the raw material. We used a crude casein obtained from Lactic Casein Importers Ltd. Remarks made here concerning this medium may not be applicable to the medium as prepared in Adams & Hendee's laboratory.

Inoculum. A 5 hr. culture (50 ml.) grown on ordinary meat broth medium containing meat was used for every litre of casein medium.

Time of growth. λ -Enzyme activity was already very marked after 4 hr. at 37°, reached a maximum at 5 hr. and stayed fairly constant up to 24 hr. Standard conditions: 5 hr. at 37°.

Strain. A few different strains of *Cl. welchii* Type B were tried, but most of the experiments quoted here were done with a strain (CN1990, Wellcome Laboratories) isolated by Mrs J. M. Barnes in the spring of 1946 from the intestine of a lamb dead from a typical lamb dysentery infection.

Appearance of organisms. Stained culture smears of *Cl. welchii* Type B grown on Adams & Hendee's (1945) medium often presented a remarkable atypical appearance. The organisms were often unusually large and unevenly stained. Frequently, although not invariably, spores were seen, sometimes in very large numbers, even in the 5 hr. cultures. The occurrence of spores seemed to be unrelated to the fact that this medium promotes λ -enzyme formation. When other types of *Cl. welchii* were grown on this medium, the appearance of the organisms was normal.

RESULTS

Purification of λ -enzyme

The first attempts at purification were made using culture filtrates obtained on meat media. The precipitates obtained by adding ammonium sulphate (600 g./l.) did not dissolve readily in water, as our experience with *Cl. welchii* Type A had led us to expect. Instead the formation of a peculiar jelly which could be dissolved on warming at 37° was noted on several occasions. After dialysing against tap water, the material was readily soluble, but dialysis resulted in a marked increase in activity against azocoll (50–80 %) as if an inhibitor had been removed. Nutrient broth did not inhibit the action of λ -enzyme on azocoll so it appears that an inhibitor may be formed during the growth of the organism. These effects were not observed with culture filtrates obtained on the casein medium and the point was not pursued. The λ -enzyme could not be adsorbed on calcium phosphate (used for the purification of collagenase) nor could it be purified by the nucleic acid method which van Heyningen & Bidwell (1948) applied to *Cl. welchii* α -toxin.

Ammonium sulphate fractionation. The best preparation obtained by a series of three fractionations with ammonium sulphate when meat media were used contained 20–24 R units of λ -enzyme/mg. of non-dialysable nitrogen (NDN) representing a purification of only four- or five-fold. When the casein medium was used, the crude starting material already contained about 20 R units of λ -enzyme/mg. NDN and moreover, by the same procedure of ammonium sulphate fractionation, a further purification of 15- to 30-fold could be attained.

The method finally adopted was as follows:

Culture filtrates obtained after 5 hr. growth on casein medium were clarified by filtration through paper pulp with the aid of Hyflo Supercel (Johns-Manville Ltd.), sterilized by passing through bacteriological filter candles, and $(\text{NH}_4)_2\text{SO}_4$ (600 g./l.) stirred in. After standing overnight at 2–4°, as much as possible of the rather small precipitate was skimmed from the surface and the rest collected by filtration

using a small amount of Hyflo Supercel. The precipitate may be dialysed directly against running tap water. After overnight dialysis, the material was cooled to about 2° in an ice-water bath, 2 vol. ice-cold 51 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution added very slowly with stirring, left overnight at 2–4° and the precipitate collected by filtration with the aid of Hyflo Supercel. The dialysis and precipitation with 2 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ was repeated once. After a final dialysis, the material was usually preserved by drying from the frozen state.

An illustrative protocol is given in Table 1 which represents an average purification, but not the best we attained. Table 2 shows that λ -antigen is retained through the stages of purification.

The purest preparation so far obtained contained 1630 *R* units/mg. non-dialysable nitrogen; overall yield was 42 %. The corresponding dried preparation contained 116 *R* units/mg.

For comparison, the standard dried preparation (0.2 *R* unit/mg.) contained 5 *R* units/mg. non-dialysable nitrogen; preparations made on ordinary meat broth medium were even more impure (approx. 1 *R* unit/mg. non-dialysable nitrogen). Incidentally to this work, the ϵ -toxin of *Cl. welchii* has been considerably purified, occurring in the same purified

fraction as the λ -enzyme, although, had this been the object of the work, a culture filtrate from *Cl. welchii* Type D would probably have been chosen as starting material. The ϵ -toxin in purified preparations appeared to be fully activated, possibly by the λ -enzyme, since the test dose was not decreased by trypsin.

Properties of λ -enzyme

pH stability. A partially purified λ -enzyme preparation (750 *R* units/ml.; 9000 λ combining-power units/ml.; 420 *R* units/mg. NDN) was diluted 1/10 in 1 % (w/v) NaCl; samples were adjusted to varying pH values as shown in Table 3 using 0.1 *N*-NaOH or 0.1 *N*-HCl. Each, as prepared, was heated for 30 min. at 37°, then cooled and re-adjusted to pH 7. A precipitate was obtained in the sample at pH 4. The control preparation was kept at pH 7 and room temperature. The results are shown in Table 3. The [E.T.] casein value for the preparation heated at pH 7 is slightly higher than would have been expected from comparison with the other activities of the same preparation, but on the whole the activities are remarkably parallel. There is some suggestion that at pH values above 7, the active

Table 1. Purification of λ -enzyme

Stage no.	Description	λ -enzyme			
		<i>R</i> units/ml.	Stage yield (%)	<i>R</i> units/mg. NDN*	Overall purification† mg. NDN*/ml.
1	Crude culture filtrate (A)	16.6	—	46	—
	23 l. A precipitated with $(\text{NH}_4)_2\text{SO}_4$ (600 g./l.); ppt. dissolved in water (B; 410 ml.)	539	67	207	4.5
	395 ml. B dialysed overnight (C; 730 ml.)	243	84	—	—
2	725 ml. C + 2 vol. 51 % (w/v) $(\text{NH}_4)_2\text{SO}_4$; ppt. dissolved in water (D; 228 ml.)	740	95	—	—
	225 ml. D dialysed overnight (E; 295 ml.)	508	89	674	14.7
	50 ml. E + 2 vol. 51 % (w/v) $(\text{NH}_4)_2\text{SO}_4$; ppt. dissolved in water (F; 27.0 ml.)	865	90	—	—
	26.5 ml. F dialysed overnight (G; 30 ml.)	685	90	835	18.6
					0.804

* NDN = non-dialysable nitrogen.

† Purification expressed in terms of non-dialysable nitrogen by the ratio $\frac{\text{Activity/mg. NDN of product}}{\text{Activity/mg. NDN of starting material}}$.

Table 2. The comparison of the enzyme activity against azocoll (in *R* units/ml.) and the λ -antigen content (antibody combining power units/ml.) at successive stages of the purification of three different batches

Stages of purification (see Table 1)	Ratio λ units*/ml. : <i>R</i> units/ml.		
	Batch 1	Batch 2	Batch 3
Crude filtrate	6.5	8.4	5.4
1	7.6	4.3	—
2	6.1	3.4	4.6
3	8.4	7.0	7.9

* Antibody combining-power units.

enzyme is less stable than total antigen, but at acid pH values enzymic activity and combining power are destroyed in parallel.

Heat stability of λ -enzyme. A sample of the preparation that was used in the previous experiment was diluted 1/10 in borate buffer pH 7.4 and divided into seven equal parts which were heated for exactly 10 min. to varying temperatures.

Table 4 shows that the purified enzyme is very heat labile, since about 75 % of the activity was destroyed by heating to 56° for 10 min. The loss in antibody combining power was parallel to the loss in enzymic activity. After 10 min. at 50°, about 20 %

Table 3. pH stability of λ -enzyme

(Each sample of enzyme was adjusted to a particular pH, heated 30 min. at 37°, cooled and readjusted to pH 7 before test.)

		Enzyme activities after treatment				
Substrate	...	Azocoll		Casein	Haemoglobin	Gelatin
pH		<i>R</i> units/ml.	λ units/ml.*	[E.U.] casein $\times 10^6$ /ml.	[E.U.] Hb $\times 10^6$ /ml.	Test doses/ml.*
4		27	294	350	200	33
5		61	720	—	—	—
6		70	770	—	—	—
7		66	740	1200	480	71
8		69	890	—	—	—
9		61	890	—	—	—
10		44	710	800	350	77
Control		79	870	1200	600	—

* Antibody combining-power units.

of the activity against casein and about 30 % of the activity against haemoglobin had been lost. After 10 min. at 56° the losses were 80 and about 70 % respectively, and this treatment also resulted in a loss of about 75 % of the combining power as measured by the gelatin agar test. After 10 min. at 60°, the enzyme activity in R units and the combining power (both azocoll and gelatin-agar) were all 5 % of the value of the control (20° sample).

Table 4. Effect on the enzyme activity and combining power of λ -enzyme of heating for 10 min. at various temperatures

Temp. (°)	R units/ml.	λ units/ml.*
Control (20)	70	820
45	64	710
50	50	680
56	17	160
60	3	30
70	<1	<2

* Antibody combining-power units.

Optimum pH. The experiments on the action of λ -enzyme preparation on hide powder were carried out as described for collagenase (Bidwell & van Heyningen, 1948). There was very little variation in activity over the range pH 5–8, with a very sharp drop below pH 5. The curves for λ -enzyme were similar to those obtained for the action of collagenase and the ‘secondary’ azocoll enzyme (Bidwell, 1949*a*) on hide powder. Collagen prepared by the method of Delaunay, Guillaumie & Delaunay (1949) was dissolved most rapidly by collagenase preparations at pH 6.5 (veronal-acetate-HCl buffer); the other enzymes were without effect. There was little variation in the activity of λ -enzyme preparations on casein over the range of pH 6–7.2 or on haemoglobin over the range 7–8.4.

Optimum substrate ‘concentration’ for the action of λ -enzyme preparations on hide powder. The difficulty of working with concentrated suspensions of hide

powder which had been encountered in the experiments with collagenase was greatly accentuated with λ -enzyme. Although hide powder was readily dissolved, further breakdown appeared to be slow, so that, when the higher concentrations of hide powder were used, the mixture was extremely

Fig. 2. Variation of enzyme activity with ‘concentration’ of hide powder in suspension. Collagenase, final concentration 0.28 Q unit/ml., in borate buffer, pH 7.4, — x — x —, λ -enzyme, final concentration 0.4 R units/ml., in borate buffer, pH 7, —●—●—. Varying amounts of hide powder in total volume 10 ml. buffer-enzyme mixture incubated for 1 hr. at 37°. Controls: heat-inactivated enzyme.

difficult to filter, being very ‘gelatinous’. It was feared that results might be invalidated by the enzyme reaction continuing during the filtration and therefore the experiment was repeated with the modification that after incubation the mixture was rapidly heated to 70° for 10 min. to destroy the enzyme before filtering. This procedure resulted in slightly higher values in the controls, as might be expected, but the result did not differ qualitatively from that previously obtained for λ -enzyme. Fig. 2

shows that the results with λ -enzyme were very different from those obtained with collagenase, since even the highest concentration of hide powder attainable did not appear to be near the optimum for λ -enzyme.

Activity curve. Owing to the difficulty in filtering the digested hide powder after the action of the λ -enzyme and the fact that no optimum 'concentration' of substrate was reached, no attempt was made to determine an activity curve for the action of λ -enzyme on hide powder.

The action of λ -enzyme preparations on gelatin. *Cl. welchii* Type B filtrates contain a substance which attacks gelatin so that it no longer gives a precipitate on the addition of an acid solution of mercuric chloride. This substance is probably identical with λ -enzyme (Oakley *et al.* 1948). λ -Enzyme preparations attack gelatin with the formation of split products which are not precipitated on the addition of acidified phosphotungstic acid under the conditions described for collagenase (Bidwell & van Heyningen, 1948). The actions of partially purified preparations of collagenase, the 'secondary' enzyme and λ -enzyme on gelatin, as measured by the non-precipitable nitrogen method, are all considerably increased in the presence of horse serum.

It appeared possible that our enzyme preparations might contain a kinase capable of activating serum protease (compare streptokinase, Christensen, 1945, 1946; Christensen & MacLeod, 1945), but experiments designed to test this were all negative. The effects observed by this method may largely be due to yet another enzyme, possibly a polypeptidase which is activated by normal horse serum and is a relatively poor antigen.

Substrate specificity

Casein and haemoglobin. Substrates used were 2.5 % casein in veronal-acetate-HCl buffer, pH 7, and urea-denatured haemoglobin (Anson, 1938). 'Tyrosine equivalents' were determined on the filtrates by the method of Anson (1938). Table 5 shows that λ -enzyme preparations attacked both substrates; the results obtained with collagenase and the 'secondary' enzyme are included for comparison.

The question whether the splitting of casein and haemoglobin by λ -enzyme preparations can be regarded as due to λ -enzyme has not been satisfactorily settled. From a comparison of the activities of several samples at different stages of purification when tested against the different substrates it appeared that more than one enzyme must be involved. Normal horse sera and specific antisera inhibited the action of the preparations on casein and haemoglobin; we therefore tried to tackle the problem by serological methods. The results of attempts at the quantitative determination of inhibitory activity of sera using casein as substrate were however very inconsistent and were even less promising with haemoglobin.

Silk proteins. Raw silk or silk fibroin (400 mg.) cut into small pieces were used in a total volume of 20 ml. Where cysteine was used the final concentration was 0.004M (pH adjusted to 7). Collagenase (10 Q units/ml.) and 'secondary' enzyme (5 'Q' units/ml.) preparations had no effect on raw silk in the absence of cysteine; with added cysteine there was an increase in filtrate nitrogen in 20 hr. at 37°. λ -Enzyme (10 R units/ml.) had a definite action on raw silk which was inhibited by cysteine (Table 6). None of the enzymes digested silk fibroin, either with or without cysteine, so the action on raw silk was probably due to digestion of seracin.

Clupein. Collagenase and λ -enzyme preparations showed an extremely small activity which was enhanced by cysteine; the more active preparation (λ) split only about 3 % of the available bonds after 20 hr. at 37°. The final concentration of enzyme in the solution was approx. 8Q or R units/ml. The 'secondary' enzyme was completely inactive, even in the presence of cysteine.

For comparison either collagenase or λ -enzyme in a concentration of about 0.3Q or R units/ml. will digest nearly 25 % of the hide powder present in 2.5 % suspension in 1 hr. at 37°.

Peptidase activity. Partially purified λ -enzyme preparations liberated titratable groups from gelatin, but their activity was even weaker than that of collagenase (Bidwell & van Heyningen, 1948). The relative weakness of λ -enzyme in breaking down the

Table 5. Action of collagenase, 'secondary' enzyme and λ -enzyme on casein and haemoglobin

(5.0 ml. substrate and 1 ml. enzyme solution were incubated at 37° for the times shown. 10 ml. 0.3M-trichloroacetic acid were added and the mixtures filtered after 0.5 hr. Controls: precipitant added before enzyme.)

Enzyme	Activity against azocoll (Q or R units)	Substrate	Digestion time (min.)	Tyrosine in 5 ml. digestion products (m-equiv. $\times 10^4$)
Collagenase	300	Casein	30	2.7
	450	Haemoglobin	30	2.0
'Secondary' enzyme	300	Casein	30	-0.4
	350	Haemoglobin	30	+0.8
λ -Enzyme	30	Casein	10	9.1
	45	Haemoglobin	10	10.7

Table 6. *The action of collagenase 'secondary' enzyme and λ -enzyme on silk proteins*

Enzyme	Cysteine	Substrate	N liberated (mg./5 ml. filtrate)		
			Active enzyme	Heat-inactivated enzyme	Difference
Collagenase	-	Raw silk	0.145	0.124	0.021
	+	Raw silk	0.896	0.684	0.212
'Secondary'	-	Raw silk	0.075	0.070	0.005
	+	Raw silk	0.625	0.345	0.280
λ -Enzyme	-	Raw silk	0.599	0.128	0.471
	+	Raw silk	0.619	0.505	0.114
Collagenase	-	Fibroin	0.127	0.103	0.024
	+	Fibroin	0.469	0.447	0.022
'Secondary'	-	Fibroin	0.065	0.055	0.010
	+	Fibroin	0.325	0.315	0.010
λ -Enzyme	-	Fibroin	0.110	0.098	0.012
	+	Fibroin	0.470	0.474	-0.004

Table 7. *Effect of cysteine on the digestion of hide powder by (a) λ -enzyme, (b) collagenase*

(100 mg. hide powder, 9 ml. borate buffer and 1 ml. enzyme maintained at 37° for 0.5 hr. Filtered. Total N determined in filtrate.)

Enzyme	Cysteine	N liberated (mg./ml. filtrate)			Inhibition (%)
		Active enzyme	Heat-inactivated enzyme	Difference	
λ -Enzyme (0.21 R units/ml.)	-	0.637	0.166	0.471	-
	+	0.235	0.212	0.023	> 90
Collagenase (0.17 Q units/ml.)	-	0.497	0.189	0.308	-
	+	0.397	0.225	0.172	44

gelatin has already been noted in connexion with the experiments on hide powder (see also Walbum & Reymann, 1934).

Enzyme inhibitors

Metallic ions. The activity of λ -enzyme preparations in azocoll tests was almost completely destroyed by heating 0.5 hr. at 37° with 0.001M solutions of copper or mercury salts; under the same conditions, ferric iron had no effect, while ferrous iron decreased the activity to about 50 %. The digestion of hide powder by λ -enzyme previously dialysed against distilled water was unaffected by the addition of calcium or magnesium ions in final concentrations of 0.1 or 0.001M.

Cysteine. Equal volumes of enzyme preparation and 0.1M-cysteine (adjusted to pH 7) were allowed to stand together for 10 min.; control mixtures were made containing saline instead of cysteine.

Table 7 shows that both λ -enzyme and collagenase are markedly inhibited by cysteine. It seems probable that the slight action of collagenase preparations on casein, haemoglobin, seracin and clupein which is increased in the presence of cysteine is not due to collagenase itself, but perhaps to traces of Maschmann's 'anaerobiase' (Maschmann, 1938a, b).

Weil & Kochololaty (1937) and Weil, Kochololaty & Smith (1939) stated that the gelatinase of *Cl.*

welchii was markedly activated by cysteine + iron. Both collagenase and λ -enzyme showed decreased activity in dissolving hide powder in the presence of the concentrations of cysteine and iron used by Weil *et al.* (1939).

Iodoacetic acid (final concentration 1 mg./ml.) had a slight inhibitory effect on both enzymes. λ -Enzyme was slightly inhibited by cyanide, but collagenase was not affected at the concentration used (enzyme preparations were allowed to stand 0.5 hr. at room temperature with 0.01M-cyanide); concentration of cyanide during activity test was approx. 0.0002M).

Citrate. The inhibitory effect of citrate was tested using purified collagenase, before and after alkali treatment, and purified λ -enzyme, all three being diluted to approximately the same activity against azocoll (5Q or R units/ml.). Table 8 shows that neither collagenase nor the secondary enzyme was appreciably affected by a final concentration of 0.1M-citrate, whereas λ -enzyme was 75 % inhibited.

'Trypsin inhibitors.' Soybean trypsin inhibitor was without effect on any of the three enzymes when tested against azocoll: equal volumes of enzyme (diluted to approx. 5Q or R units/ml.) and inhibitor solution (5 or 0.5 mg./ml.) were mixed, left 0.5 hr. and the activity against azocoll then determined and shown to be the same as when no soybean inhibitor was present. Pancreatic trypsin inhibitor was tested

Table 8. *The effect of citrate on the activity of collagenase, the 'secondary' enzyme and λ -enzyme in disintegrating hide powder*

(250 mg. hide powder and 10 ml. enzyme mixture maintained at 37° for 1 hr. (1) With citrate: 7 ml. borate buffer pH 7 + 2 ml. 0.5M-citrate pH 7 + 1 ml. enzyme. (2) Without citrate: 9 ml. borate buffer pH 7 + 1 ml. enzyme. (3) Enzyme controls: corresponding heat-inactivated enzymes.)

Enzyme	mg. N/ml. filtrate		Difference (active enzyme control)		Inhibition by citrate (%)
	With citrate	Without citrate	With citrate	Without citrate	
Collagenase	1.98	2.04	1.62	1.58	0
Collagenase control	0.36	0.46			
'Secondary' enzyme	2.07	2.10	1.72	1.63	0
'Secondary' control	0.35	0.47			
λ -Enzyme	0.74	2.12	0.40	1.64	75
λ -Enzyme control	0.34	0.48			

Table 9. *Comparison of the properties of collagenase, 'secondary' enzyme and λ -enzyme*

Observation	Collagenase	'Secondary' enzyme	λ -Enzyme
Occurrence in culture filtrates of types of <i>Cl. welchii</i>	Types A, C, E, some D	?	Types B, E, some D
Action on substrates:			
Native collagen	+	-	-
Hide powder, gelatin	+	+	+
Casein, haemoglobin	(-)	-	+
Seracin	(-)	(-)	+
Clupein	-	-	-
Activity <i>in vivo</i>	Various activities ascribable to attack on collagen (Oakley <i>et al.</i> 1948)	Probably none	None known
Optimum pH	pH 6.5 (collagen) pH 6.0-7.5 (hide powder)	pH 5.0-7.5	pH 6.0-7.5
Effect of acid	Unstable at pH < approx. 5	Unstable at pH < approx. 5	Unstable at pH < approx. 5
Effect of alkali	Unstable at pH > approx 8.5	Appears in collagenase preparation at pH between 9 and 10.5. Destroyed at pH > 10.5	Unstable at pH > 9
Effect of heat	Destroyed in 10 min. at 56°	Appears in collagenase preparations at 50°, destroyed in 10 min. at 60° in borate buffer, probably more stable in distilled water	95 % destroyed in 10 min. at 60°
Inhibition by:			
Cysteine	+	?	+
Cyanide	-	?	+
Citrate	-	-	+
Normal horse serum	-	-	+

in a similar way, but owing to insufficient material being available only the lower concentration (0.5 mg./ml.) was used, and the effect on the secondary enzyme was not tested. λ -Enzyme was definitely not affected. The activity of the collagenase preparation was decreased about 10 %, but it was unfortunately impossible to repeat the experiment as no more inhibitor was available.

A comparison between the properties of λ -enzyme, collagenase and the 'secondary' enzyme (which appears in preparations of collagenase which have been treated with alkali or heat) is summarized in Table 9.

DISCUSSION

Maschmann (1937, 1938*a, b*) obtained a protease (anaerobiase) from culture filtrates of *Cl. welchii* which could split casein and gelatin (optimally only in the presence of cysteine). Clupein and peptone were also split if a further, unknown activator in addition to cysteine was present. Anaerobiase was inhibited by normal serum, but cannot be identified with λ -enzyme, since the latter is inhibited by cysteine even more markedly than is collagenase. It seems unlikely that any of our preparations has contained more than, at most, a trace of anaerobiase.

The enzyme we have called collagenase seems to be the same as the 'specific gelatinase' (collagenase) of Maschmann.

Maschmann (1938c) described a 'new' proteinase of *Clostridium sporogenes* (*B. sporogenes*) which appears to be similar to λ -enzyme in some of its biochemical properties, e.g. inhibition by citrate and normal horse serum; partial inhibition by cysteine and cyanide; optimum pH; lability to acid pH and its comparatively weak gelatinase action when assessed by a method depending on the degree of chemical degradation of the substrate.

An enzymic digest of casein probably simulates the natural medium of a lamb dysentery infection rather more closely than the conventional meat medium, so that a comparison of toxin production on the two types of media is of particular interest.

When grown on the ordinary laboratory meat media, *Cl. welchii* Type B usually produces high β -toxin and low ϵ -toxin values. (The average of thirteen different routine batches was 0.57 ml. = 1 ϵ unit of antitoxin; 0.58 ml. = 10 β units). With Adams & Hendee (1945) medium β values were very low (> 1.0 ml. = 2 β units) and ϵ values relatively high (usually 0.2–0.4 ml. = 5 ϵ units). *Cl. welchii* Type D is routinely grown on meat broth medium containing meat particles; the mean of seventeen such batches of Type D toxin gave 0.39 ml. = 5 ϵ units, i.e. Type B grown on casein medium can produce as much ϵ -toxin as Type D. On a few occasions Type D was grown on casein medium; the ϵ values were no higher than observed with Type B. It seems likely that the low β values were due to destruction of β -toxin by a proteolytic enzyme (such as λ -enzyme) since when a strain of Type C (which does not produce λ -enzyme) was grown, a titre of about 0.1 ml. = 10 β units was obtained, and the growth of Type B in the presence of normal horse serum (which inhibits λ -enzyme) resulted in the production of small amounts of β -toxin. Thus, apart from the high λ -enzyme content of filtrates obtained on the casein medium, the content of β - and ϵ -toxins is similar to

that obtained on meat media with Type D strains rather than Type B. Unpublished experiments suggest that the unusual distribution obtained on Adams & Hendee's (1945) medium arises from some factor in whole pancreas which is not extracted by the usual processes for preparing 'pancreatic extract'.

SUMMARY

1. The partial purification and properties of the λ -enzyme of *Clostridium welchii* Type B are described. In a special medium (pancreatic digest of casein) *Cl. welchii* Type B produced high yields of λ -enzyme and ϵ -toxin, but little or no β -toxin.

2. Purification of λ -enzyme led to parallel purification of λ -antigen, and the two are considered to be identical.

3. λ -Enzyme was purified about 40 times in terms of the non-dialysable nitrogen in the crude filtrate from the casein medium in which it was already about 40 times as pure as in ordinary meat broth media.

4. The enzyme preparations attacked hide powder, gelatin, casein, haemoglobin and seracin, but had no effect on native collagen.

5. The optimum pH was pH 6.0–7.5.

6. The enzyme was destroyed at values below pH 5 or above 9 or by heating to 60° for 10 min.

7. Citrate inhibited the enzyme strongly, but there was no evidence for activation by calcium or magnesium ions. The enzyme was slightly inhibited by iodoacetic acid and cyanide and strongly inhibited by cysteine.

8. Its properties were compared with those of collagenase and the 'secondary' enzyme of *Clostridium welchii* Type A.

I am grateful to Mrs J. M. Barnes and Miss H. E. Ross for assistance and advice in the bacteriological techniques, to Mrs I. Batty for determinations of β - and ϵ -toxin, to Dr C. L. Oakley and Miss G. H. Warrack for many helpful discussions and to Miss B. M. Sievwright for technical assistance. I wish to thank Dr A. Delaunay for a gift of rat-tail collagen and Dr F. O. Howitt for the silk proteins.

REFERENCES

- Adams, M. H. & Hendee, E. D. (1945). *J. Immunol.* **51**, 249.
 Adams, M. H., Hendee, E. D. & Pappenheimer, A. M. (1947). *J. exp. Med.* **85**, 701.
 Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
 Batty, I. & Glenny, A. T. (1947). *Brit. J. exp. Path.* **28**, 110.
 Bidwell, E. (1949a). *Biochem. J.* **44**, 28.
 Bidwell, E. (1949b). *Biochem. J.* **44**, vi.
 Bidwell, E. & van Heyningen, W. E. (1948). *Biochem. J.* **42**, 140.
 Christensen, L. R. (1945). *J. gen. Physiol.* **28**, 363.
 Christensen, L. R. (1946). *J. gen. Physiol.* **30**, 149.
 Christensen, L. R. & MacLeod, C. M. (1945). *J. gen. Physiol.* **28**, 559.
 Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed. London: Baillière, Tindall and Cox.
 Delaunay, M., Guillaumie, M. & Delaunay, A. (1949). *Ann. Inst. Pasteur*, **76**, 16.
 Diehl, H. S. (1919). *J. infect. Dis.* **24**, 347.
 Glenny, A. T., Llewellyn-Jones, M. & Mason, J. H. (1931). *J. Path. Bact.* **34**, 201.
 Haines, R. B. (1931). *Biochem. J.* **25**, 1851.
 Haines, R. B. (1932). *Biochem. J.* **26**, 323.
 Logan, M. A., Tytell, A. A., Danielson, I. S. & Griner, A. M. (1945). *J. Immunol.* **51**, 317.
 Maschmann, E. (1937). *Biochem. Z.* **295**, 1.
 Maschmann, E. (1938a). *Biochem. Z.* **295**, 351.
 Maschmann, E. (1938b). *Biochem. Z.* **297**, 284.

- Maschmann, E. (1938c). *Biochem. Z.* **300**, 89.
 Merrill, A. T. & Clark, W. M. (1928). *J. Bact.* **15**, 267.
 Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
 Mueller, J. H. (1935). *J. Bact.* **29**, 383.
 Oakley, C. L., Warrack, G. H. & van Heyningen, W. E. (1946). *J. Path. Bact.* **58**, 229.
 Oakley, C. L., Warrack, G. H. & Warren, M. E. (1948). *J. Path. Bact.* **60**, 498.
 Pope, C. G. (1938). *Brit. J. exp. Path.* **19**, 245.
 Pope, C. G. (1939a). *Brit. J. exp. Path.* **20**, 132.
 Pope, C. G. (1939b). *Brit. J. exp. Path.* **20**, 201.
 van Heyningen, W. E. & Bidwell, E. (1948). *Biochem. J.* **42**, 130.
 Walbum, L. E. & Reymann, G. C. (1934). *J. Path. Bact.* **39**, 669.
 Weil, L. & Kocholaty, W. (1937). *Biochem. J.* **31**, 1255.
 Weil, L., Kocholaty, W. & Smith, L. D. (1939). *Biochem. J.* **33**, 893.

Absorption and Excretion of the Mono-sodium Salt of Thyroxine Labelled with Radioactive Iodine

By J. C. CLAYTON, AUDREY A. FREE, J. E. PAGE, G. F. SOMERS AND E. A. WOOLLETT
Research Division, Glaxo Laboratories Ltd., Greenford, Middlesex

(Received 7 December 1949)

There has been doubt in the past as to the efficacy of thyroxine administration by the oral route; some workers have even considered that thyroxine is inactive by mouth. A preference has been shown for the administration of dried glandular extracts, in which thyroxine is bound to protein or polypeptide. It was believed that thyroxine, being a relatively insoluble substance, was poorly absorbed from the gastro-intestinal tract (Harrington, 1933). However, Schittenhelm & Eisler (1932) and Barnes & Bueno (1933), using Thiry-Vella fistulas in dogs, were able to show that thyroxine was absorbed from the closed intestinal loops. There is some evidence that the form in which thyroxine is administered has a bearing on its activity after oral administration, and Thompson, Thompson & Dickie (1933) found that thyroxine by this route was most active as the sodium salt and that it can be used successfully in the treatment of myxoedema (cf. also Hart & MacLagan, 1950). Bailey, Bartlett & Folley (1949) have recently shown that sodium L-thyroxine by mouth also stimulates milk secretion in lactating cows.

Following the development of an improved synthesis of thyroxine (Borrows, Clayton & Hems, 1949; Clayton & Hems, 1950) it became of interest to obtain data on its absorption after oral dosage. We have prepared and administered the mono-sodium salt of DL-thyroxine, labelled with radioactive ^{131}I in the 3'- and 5'-positions, to rats and cats in small doses with a view to determining its distribution and excretion.

The earlier work on the fate of thyroxine in the body has been reviewed by Elmer (1938) and Leblond (1948). In most of the earlier experiments the distribution of thyroxine was studied either by chemical estimation of iodine or by biological assays which, in view of low tissue concentrations and lack of

specificity, were far from satisfactory. Such studies have been considerably facilitated by the advent of labelled thyroxine, which was first prepared by Joliot, Courier, Horeau & Süe (1944a; see also Horeau & Süe, 1945). These investigators carried out a few experiments on rabbits (Joliot *et al.* 1944a, b; Joliot, 1945). The first detailed studies are due, however, to Gross & Leblond (1947), who gave relatively large intravenous injections of labelled thyroxine to rats.

We have administered physiological doses by the oral, subcutaneous and intravenous routes to both rats and cats. A preliminary account of our results has been published (Clayton, Free, Page, Somers & Woollett, 1949).

EXPERIMENTAL

Preparation of labelled thyroxine

The labelled thyroxine was prepared from 3:5-diiodo-thyronine by a modification of the method of Clayton & Hems (1950). To a solution of ^{131}I (1 mc.) as iodide in buffered bisulphite solution (pH 7; approx. 1 ml.), 2.3 mg. of NaI were added. The solution, in a centrifuge tube, was overlaid with 1 ml. of ether, and one drop of N-HCl and excess 0.3% H_2O_2 solution (approx. 0.5 ml.) were added. After occasional shaking for 2 hr., the lower iodine-free layer was removed by means of a pipette. DL-3:5-Diiodothyronine (2 mg.) in 0.06 ml. of 33% (w/v) aqueous ethylamine solution was added and the mixture shaken carefully when the iodine colour was discharged. The ether layer was removed by blowing a stream of air over the surface, and the aqueous layer was diluted with 1 ml. of water and adjusted to between pH 4 and 5 by the addition of glacial acetic acid. The precipitated thyroxine was spun down in a centrifuge and washed with successive amounts (1 ml.) of distilled water until the washings contained less than 5% of non-thyroxine ^{131}I as demonstrated by *n*-butanol extraction of the aqueous washings (Blau, 1935). About 14 washings were