CLVII. STUDIES ON THE PROTEINASE OF *CLOSTRIDIUM HISTOLYTICUM*

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THE proteolytic enzymes have been widely investigated during recent years. Studies on animal and plant proteinases have yielded clear, relatively uncontradictory results, but work in the field of bacterial proteinases has not been so systematic or so productive of concordant results. A comprehensive review of the latter has been presented by Haines [1934].

A considerable amount of work has been done on the proteolytic micro-organism, *Clostridium histolyticum*, belonging to the group of spore-forming anaerobes. Owing to its extraordinary proteolytic activity, this organism is especially useful for enzymic studies. Proteolytic processes furnish the energy required for its growth; in all probability the proteins of the culture medium are decomposed by enzymes secreted by the organism, the resulting breakdown products then serving as energy sources. Because of the exogenous character of these processes, it is possible to obtain highly active enzyme preparations.

Enzyme solutions for use in the present study were obtained by inoculating protein culture media with a pure strain of *Cl. histolyticum* (American Type Culture Collection, No. 4972), and after a period of anaerobic incubation, filtering through a Berkefeld filter to remove bacteria and tissue debris. Since all the known proteinases can be characterized by their pH optima and activation behaviour, careful studies of these factors were made. It was found that the proteinase of *Cl. histolyticum* has an optimum at pH 7, which does not agree with the findings of earlier workers [Blanc & Pożerski, 1920; Dernby & Blanc, 1921; Bessey & King, 1934]. In this respect, it differs from other known proteinases. Thus the optimum pH for pepsin is near 2, for cathepsin 4, for pancreatic proteinase 9, and for papain and yeast proteinase about 5.

With respect to activation behaviour, the proteinase of *Cl. histolyticum* also shows a unique character. It is indifferent towards enterokinase, the activator of tryptic proteinase, and can therefore not be classed as a tryptic type of enzyme. Sulphydryl compounds, well known as the activators of papain and catheptic proteinases, however, strongly activate the proteinase of *Cl. histolyticum*. Little work appears in the literature on the activation of bacterial proteinases. Gorbach & Ulm [1935] reported that HCN activates the proteinase of *Mammococcus gorini*. This observation, however, is contradicted by the work of Gorini *et al.* [1933].

Our investigation on the activation of the proteinase of *Clostridium histolyticum* leads to the conclusion that sulphydryl groups possess especial importance, since marked activation effects are observed only in their presence. The activation produced by sulphydryl groups can be considerably increased by the addition to the reaction mixture of heavy metal ions, such as Fe⁺⁺, Co⁺⁺, Mn⁺⁺, Ni⁺⁺ and Cu⁺⁺. The effect of the heavy metal ions seems to be a catalytic one, since a very low concentration (2.5×10^{-4} N) is sufficient to produce the maximum effect. (Co⁺⁺ is an exception.) The metal must be in the ionized form; thus iron

in complex combination (e.g. potassium ferrocyanide) had no effect. Activation effects with very low concentrations of Fe^{++} and Mn^{++} were obtained only in experiments which were carried out without buffer, but in which the $p\text{H}$ nevertheless remained constant because of the buffering action of the protein substrate. In citrate-phosphate buffer (McIlvaine), which possesses a wide $p\text{H}$ range and is well suited to such studies, higher Fe^{++} and Mn^{++} concentrations were necessary to give maximum activation. Ni^{++} and Cu^{++} , however, give maximum activation in very low concentrations, whether buffer is present or not. The maximum activation with Co^{++} was obtained only with higher concentrations and in the presence of buffer; in the absence of buffer the effect was considerably lower.

Since heavy metal ions have a catalytic effect on the oxygen uptake of sulphhydryl compounds [Michaelis, 1931; Elliott, 1930], the question arose as to whether the sulphhydryl-heavy metal system was exerting its activating effect by oxidizing the enzyme molecule (by activating molecular oxygen), or by reducing the enzyme, being itself simultaneously oxidized. To approach this question, the activation behaviour was studied under aerobic and anaerobic conditions. In pure oxygen, there was no activation by cysteine- Fe^{++} , owing to complete oxidation of the sulphhydryl group. In air some activation occurred. When the experiments were carried out in hydrogen, or *in vacuo*, however, a much greater activation was observed. Therefore, the proteinase of *Cl. histolyticum* can be maximally activated only under anaerobic conditions, a fact which agrees well with the anaerobic nature of this micro-organism.

Although the activation effects are influenced by the aerobic or anaerobic conditions of the experiments, the natural or so-called initial activity (i.e. the activity without added activator) is not affected by these factors. Hydrogen or oxygen, even when activated with palladium, has no marked activating or inhibiting effect on the initial activity of the enzyme.

Investigations on papain, cathepsin [Bersin, 1933; Mothés, 1933; Maschmann & Helmert, 1933] and urease [Sumner & Poland, 1933], have made it probable that the active group of these enzymes is a sulphhydryl radical. Our findings make it appear unlikely that the active group of the proteinase of *Cl. histolyticum* is the sulphhydryl group. Thus, compounds like iodoacetic acid which destroy sulphhydryl groups had no effect on the initial activity of this proteinase. H_2O_2 in low concentration likewise did not affect the initial activity, although higher concentrations produced an irreversible destruction of the enzyme.

These negative observations indicate a difference between the activation mechanisms of the papain type and those of the *Cl. histolyticum* type of proteinase. This difference may be partly explained by the further observation that preparations of the bacterial proteinase which have been purified show a negative nitroprusside reaction, indicating the absence of sulphhydryl groups. A possible working hypothesis for the activation of this proteinase is that the combination of sulphhydryl group and heavy metal ions acts as a catalyst in reducing the as yet unknown active group of the enzyme.

When the added activator is removed from the enzyme by dialysis, the activation disappears and only the initial activity remains. Addition of fresh activator to the dialysed solution restores the original maximum activity. The activation is independent of the purity of the enzyme preparation; purified enzyme was activated to the same degree as was the crude enzyme solution. Variation in the protein substrate, with which the enzymic activity is determined, likewise does not change the activation picture; neither does the constitution of the medium in which the bacteria are grown.

That the proteinase produced is of extracellular nature, is shown by the fact that bacteria-free filtrates contain almost all the proteinase activity shown by the original, unfiltered incubation mixture. A similar observation was made by Virtanen & Tarnanen [1931] on *Pseudomonas liquefaciens*.

A study of the relation between the growth of *Clostridium histolyticum* and the secretion of proteinase shows that the proteinase activity runs parallel with the growth, and that the maximum in each case is reached in about 24 hr. This maximum is followed by a parallel decrease in the number of living bacteria and proteinase activity. The cause of this decrease in proteolytic activity requires further study. The ereptic activity, on the other hand, continues to increase even after maximum bacterial growth has been reached. It was possible to show that the increase in the ereptic activity is not due to autolysis of the bacteria. It seems rather that after a certain incubation period, when the lower protein molecules have been decomposed, the remaining bacteria adapt themselves by producing erepsin, which is capable of decomposing the lower molecular weight protein breakdown products.

EXPERIMENTAL

Preparation of Clostridium histolyticum proteinase

For the preparation of highly active enzyme solutions, *Cl. histolyticum* No. 4972, obtained from the American Type Culture Collection,¹ was inoculated into horse meat broth medium (National Drug Company), and incubated in a McIntosh-Fildes anaerobic jar at 37° in an atmosphere of hydrogen. The medium was boiled before inoculation to remove traces of dissolved oxygen. Unless otherwise specified, the period of incubation was 3 days. The culture was then filtered through a Berkefeld filter and the bacteria-free filtrate used directly for the enzyme studies. Unless otherwise specified, the proteolytic activity was measured by determining the increase in free amino-groups produced in a standard protein substrate (gelatin) by the Van Slyke method. The increase in amino-groups is, in general, expressed in terms of ml. 0.1 N KOH equivalent to the amino-groups liberated.

pH optimum

Since, as is shown later, citrate-phosphate buffer [McIlvaine, 1921] has no inhibiting effect on the enzymic activity, it was used in establishing the pH optimum. Measurements were made at various pH values, using 1 ml. of enzyme solution, 5 ml. of buffer, 3 ml. of 6.6 % gelatin solution (0.2 g. gelatin) previously brought to the proper pH and 2 ml. of water. Toluene, which has no effect on the enzymic activity, was used as antiseptic, and for each determination a blank run was made. The incubation period was 18 hr. at 37°. A different enzyme preparation was used in preparing similar reaction mixtures in which a portion of the water was replaced by cysteine solution (10 mg. of cysteine-HCl brought to the proper pH), or by FeSO₄ solution (0.6 ml. of 0.1 N), or by both. At the end of the incubation period the —NH₂ increase was determined in the Van Slyke apparatus, the results being expressed in Table I in terms of ml. of 0.1 N KOH. As will be seen, the optimum is at pH 7, both in the presence and absence of added activators. Fe⁺⁺ alone had an inhibitory effect; cysteine alone activated

¹. Hoogerheide [1937] recently found that *Cl. histolyticum* may dissociate into a sugar-fermenting variant, closely resembling *Cl. sporogenes*. The results reported in this paper were obtained with a strain in a dissociated stage. A number of experiments carried out with another, non-sugar-fermenting strain indicated, however, that no essential difference exists with respect to the activation mechanisms in the two cases.

moderately strongly; the cysteine-Fe⁺⁺ combination produced the highest activity. The latter is the so-called "full activity" of the enzyme.

Table I. *pH optimum of Cl. histolyticum proteinase*

(pH) ...	4.0	5.0	6.0	6.6	7.0	7.6	8.0
	Activity (ml. 0.1 N KOH)						
Activator							
None (initial)	0.0	0.57	2.08	2.25	2.36	2.20	2.07
Cysteine	0.0	1.06	2.75	—	2.87	—	2.48
Fe ⁺⁺	0.0	0.09	1.76	—	2.08	—	1.40
Cysteine-Fe ⁺⁺ (full)	0.09	0.89	2.79	3.30	3.87	3.40	3.08

Effect of enterokinase

Enterokinase solution was prepared according to the method of Waldschmidt-Leitz [1923]. 1 ml. of enzyme solution was added to 0.5 ml. of enterokinase and the mixture incubated at 37° for 30 min. to effect activation. Then 3 ml. of neutralized 6.6 % gelatin solution and water to bring the volume to 10 ml. were added. This mixture was incubated for 20 hr. at 37°, after which it was analysed for —NH₂ increase. As shown in Table II, the enterokinase had no effect on the activity of the proteinase.

Table II. *Effect of enterokinase on Cl. histolyticum proteinase activity*

Enzyme solution	Enzymic activity (ml. 0.1 N KOH)	
	Without kinase	With kinase
I	0.70	0.68
II	1.28	1.20

Effect of sulphhydryl compounds

In order to determine the effect of sulphhydryl concentration on the enzymic activity, varying amounts of cysteine-HCl were dissolved in small portions of water and brought to pH 7 with NaOH. To each of these were added 1 ml. of enzyme solution, 5 ml. of citrate-phosphate buffer (pH 7), 3 ml. of neutralized 6.6 % gelatin and sufficient water to make a total volume of 11 ml. The mixtures were incubated for 20 hr. at 37° in an atmosphere of hydrogen to avoid oxidation of the —SH group. As shown in Table III, maximum activation occurs over a rather wide range of concentration, 10 mg. of cysteine-HCl being sufficient.

Table III. *Effect of cysteine concentration on Cl. histolyticum proteinase*

Cysteine-HCl (mg.)	1.0	2.0	4.0	8.0	15.0
Activity (ml. 0.1 N KOH)	1.82	2.28	2.35	2.39	2.36

Several other sulphhydryl compounds were tested for activating effect in a similar manner, using concentrations equivalent to 10 mg. of cysteine-HCl. In the cases indicated, 0.6 ml. of 0.1 N FeSO₄ was also added. HCN activation was tested for by substituting 10 mg. of KCN, at pH 7, for the —SH compound; H₂S activation was carried out by bubbling a slow stream of H₂S through the enzyme solution for 30 min. before addition of substrate. Four enzyme solutions, obtained from separate inoculation mixtures, were used. The results (Table IV) are qualitatively the same for all four enzyme preparations. All sulphhydryl compounds tested produced activation, although to varying degrees. The iron effects will be discussed later.

Table IV. *Effect of —SH compounds on Cl. histolyticum proteinase*

Activator	Enzymic activities (ml. 0.1 N KOH)			
	I	II	III	IV
None	3.28	1.03	0.18	0.29
Cysteine	3.76	1.38	1.34	0.96
Fe ⁺⁺	2.80	1.56	0.86	—
Cysteine-Fe ⁺⁺	4.57	2.39	1.97	—
Glutathione	3.63	—	—	1.15
Glutathione-Fe ⁺⁺	4.01	—	—	—
Thiosalicylic acid	3.58	—	1.24	—
Thiosalicylic acid-Fe ⁺⁺	4.16	—	1.65	—
Thiolacetic acid	3.74	—	0.77	—
Thiolacetic acid-Fe ⁺⁺	4.11	—	1.39	—
Thio- β -naphthol	3.26	—	1.25	—
Thio- β -naphthol-Fe ⁺⁺	4.00	—	1.80	—
H ₂ S	1.81	—	—	0.84
HCN	3.19	1.18	—	0.72

In addition to the compounds shown in Table IV, the following produced no activation either in the presence or absence of iron: β -thiodiglycol, 1-benzo-4-phenylthiosemicarbazide, thiourea, NaHSO₃, vitamin C, alloxan. Contrary to the observations made on arginase [Weil, 1935; Purr & Weil, 1934], vitamin C or alloxan, in combination with iron, was shown to have a slightly inhibitory effect on the activity of the proteinase. Unlike the sulphhydryl compounds, which in the presence of Fe⁺⁺ always activated the enzyme, H₂S and HCN did not always give consistent results. The strong activation produced by thio- β -naphthol is rather striking in view of the extremely insoluble nature of this compound.

Effect of "activation time" on the activity of Clostridium histolyticum proteinase

In the activation of some enzymes (e.g. trypsin by enterokinase or cathepsin by sulphhydryl compounds) it is customary to incubate the enzyme with the activator for some time, usually 30 min. at 37°, before addition of the substrate. The activation is a time reaction and does not reach its full value unless this procedure is followed. This so-called "activation time" was found to be unnecessary in the case of *Cl. histolyticum* proteinase. To 1 ml. portions of enzyme solution were added 10 mg. of neutralized cysteine-HCl and 0.6 ml. of 0.1 N FeSO₄. The mixtures were incubated at 37° for 30, 60 and 90 min. respectively. Then buffer, substrate and water were added in the usual manner, and incubation was continued for 20 hr. in an atmosphere of hydrogen. Table V shows that the activation of the proteinase by the sulphhydryl-Fe⁺⁺ complex reaches its maximum value immediately, the preliminary activation time being superfluous. The initial activity of the enzyme used was equivalent to 1.80 ml. of 0.1 N KOH.

Table V. *Effect of activation time on Cl. histolyticum proteinase activity*

Activation time (min.)	0	30	60	90
Enzymic activity (ml. 0.1 N KOH)	3.22	3.18	3.16	3.20

Activation of Clostridium histolyticum proteinase in presence and absence of oxygen

Reaction mixtures were prepared exactly as previously described with enzyme, cysteine, Fe⁺⁺, buffer and gelatin. The mixtures were incubated for 20 hr. at 37° in air, in Thunberg tubes filled with hydrogen, in Thunberg tubes

evacuated with a Cenco Hyvac pump and in flasks into which a slow stream of oxygen was bubbled during the experiment. As shown in Table VI, maximum activating effect is observed both in hydrogen and *in vacuo*. The activity is slightly inhibited by air and completely by oxygen, apparently because of oxidation of the sulphhydryl group.

Table VI. *Activation of Cl. histolyticum proteinase in presence and absence of oxygen*

Activator	Activity (ml. of 0.1 N KOH) after incubation in			
	Air	Oxygen	Hydrogen	Vacuum
Cysteine	1.34	0.20	1.55	1.46
Cysteine-Fe ⁺⁺	1.80	0.22	2.49	2.42

Reversibility of the enzymic activation

The inhibition of the cysteine-Fe⁺⁺ activity caused by oxygen (Table VI) is reversible. To 10 ml. of enzyme were added 50 mg. of neutralized cysteine-HCl in 5 ml., 3 ml. of 0.1 N FeSO₄ and 50 ml. of buffer (pH 7). The mixture was oxygenated for 20 hr. The activity was then measured on 7 ml. portions by adding 3 ml. of 6.6% gelatin and 1 ml. of water (or 1 ml. containing 10 mg. of neutralized cysteine-HCl and 0.6 ml. of 0.1 N FeSO₄) and incubating for 20 hr. at 37° in hydrogen. Table VII shows that oxygenation reduces the activity to the initial value, and that addition of fresh activator restores almost completely the cysteine-Fe⁺⁺ activity obtained before the treatment with oxygen. The same reversibility was found in an enzyme solution which had been inactivated by dialysis. 20 ml. of enzyme solution were activated with 5 ml. of neutralized cysteine-HCl (100 mg.) and 5 ml. of 0.1 N FeSO₄. The mixture was dialysed for 4 hr. against distilled water in a cellophane bag. Initial and cysteine-Fe⁺⁺ activities were measured both before and after dialysis on portions equivalent to 1 ml. of enzyme, by adding gelatin, buffer and fresh activator where indicated, and incubating for 20 hr. in the usual manner. As shown in Table VII, dialysis reduces the activity almost to the initial value, but addition of fresh activator completely restores the original cysteine-Fe⁺⁺ activity. The dry weight of 1 ml. of the original enzyme solution was 22.5 mg. and after dialysis 3.1 mg., indicating a considerable purification. Nevertheless the activation behaviour was the same in each case.

Table VII. *Reversibility of the Cl. histolyticum proteinase activation*

Treatment	Activity (ml. 0.1 N KOH)			
	Before treatment		After treatment	
	Initial activity	Cysteine-Fe ⁺⁺ activity (in H ₂)	Remaining activity	After fresh cysteine-Fe ⁺⁺ addition (in H ₂)
Oxygenation	0.42	2.18	0.48	2.02
Dialysis	0.18	1.97	0.24	2.01

Effects of metallic ions

Experiments were carried out to determine the effects of various heavy metal ions, alone and in combination with cysteine, on the proteinase activity of *Cl. histolyticum*. Since the presence of buffer salts might interfere with the action of the metal ions, determinations were made both with and without

citrate-phosphate buffer.¹ The citrate prevents the precipitation of certain of the metals as phosphates. The pH of the unbuffered mixtures did not change appreciably during the experimental period, the maximum variation being 0.2 unit. Reaction mixtures were made up as previously described, using 1 ml. of enzyme solution, 3 ml. of 6.6% gelatin (pH 7) and 7 ml. of water; where indicated, a portion of the water was replaced by one or more of the following: 5 ml. of citrate-phosphate buffer (pH 7), 10 mg. of neutralized cysteine-HCl in 1 ml., or 0.025–0.8 ml. of 0.1 *N* solutions of heavy metal salts. The mixtures were incubated for 18 hr. at 37° in hydrogen, the —NH₂ increase being measured by the Van Slyke method.

Fig. 1² shows the results obtained with FeSO₄. When used alone, it inhibits strongly in absence of buffer, and slightly when buffer is present. (However, occasionally bacterial filtrates have been encountered whose proteinase is slightly activated by Fe⁺⁺.) In combination with cysteine, only traces of Fe⁺⁺ (0.00025 *N*) were necessary to produce maximum activation in the absence of buffer. In the presence of buffer (plus cysteine), this concentration must be increased to 0.006 *N* before maximum activation is obtained. (This is equivalent to the 0.6 ml. of 0.1 *N* FeSO₄ in 10 ml. of buffered reaction mixture customarily used in other experiments.) This difference in the presence of buffer is probably due to a decrease in the degree of ionization of the iron. In separate experiments it was found that iron in non-ionizable form (e.g. potassium ferrocyanide) had no activating or inhibiting effect on the proteinase, either in the presence or absence of cysteine.

Fig. 1

Fig. 2

○—○ Activity with cysteine + Fe⁺⁺ without buffer.
 ○---○ Activity with cysteine + Fe⁺⁺ with buffer.
 ×—× Activity with Fe⁺⁺ without buffer.
 ×---× Activity with Fe⁺⁺ with buffer.
 Line I, initial activity. Line II, activity with cysteine.

Fig. 2.

○—○ Activity with cysteine + Mn⁺⁺ without buffer.
 ○---○ Activity with cysteine + Mn⁺⁺ with buffer.
 ×—× Activity with Mn⁺⁺ without buffer.
 ×---× Activity with Mn⁺⁺ with buffer.
 Line I, initial activity. Line II, activity with cysteine.

Fig. 2 presents the results obtained with MnSO₄ and cysteine. As in the case of Fe⁺⁺, Mn⁺⁺ in combination with cysteine markedly increases the activity

¹ Veronal-HCl buffer inhibits the enzymic activity to some extent.

² For purposes of comparison, in Figs. 1–5 the initial activity (no activator present) and the activity with cysteine alone are indicated by lines parallel to the abscissa. It was found that the presence of buffer had no influence on these activities.

above the cysteine value. Here again, to produce maximum activation, a higher concentration of metallic ion is required in the presence of buffer than in its absence. Even in the absence of cysteine, manganous ions produce activation. For purposes of comparison, the initial, cysteine, Fe^{++} and cysteine- Fe^{++} activities of the enzyme solution used in this experiment were found to be equivalent to 1.00, 1.38, 1.53 and 2.57 ml. of 0.1 N KOH, respectively.

The effects of NiCl_2 and CuSO_4 , in presence and absence of cysteine, are shown in Figs. 3 and 4. The chief difference between the activations produced

Fig. 3

Fig. 4

Fig. 3.

○—○ Activity with cysteine + Ni^{++} without buffer.
 ○---○ Activity with cysteine + Ni^{++} with buffer.
 ×—× Activity with Ni^{++} without buffer.
 ×---× Activity with Ni^{++} with buffer.
 Line I, initial activity. Line II, activity with cysteine.

Fig. 4.

○—○ Activity with cysteine + Cu^{++} without buffer.
 ○---○ Activity with cysteine + Cu^{++} with buffer.
 ×—× Activity with Cu^{++} without buffer.
 ×---× Activity with Cu^{++} with buffer.
 Line I, initial activity. Line II, activity with cysteine.

Fig. 5.

○—○ Activity with cysteine + Co^{++} without buffer.
 ○---○ Activity with cysteine + Co^{++} with buffer.
 ×—× Activity with Co^{++} without buffer.
 ×---× Activity with Co^{++} with buffer.
 Line I, initial activity. Line II, activity with cysteine.

by Ni^{++} and Cu^{++} and those produced by Fe^{++} and Mn^{++} is that the former two produce their maximum activation, independently of the presence or absence of buffer, in very low concentrations and in the presence of cysteine. Increasing

the concentration of the metal ion decreases the activation. Thus with 0.006 *N* Ni^{++} plus cysteine, the activity is depressed to the initial value; with 0.006 *N* Cu^{++} plus cysteine, the enzyme is almost completely inhibited. Both Ni^{++} and Cu^{++} , in absence of cysteine, activate slightly in very low concentrations, but as this is increased, they exert an increasing inhibiting effect. For purposes of comparison, the initial, cysteine, Fe^{++} and cysteine- Fe^{++} activities of the enzyme solution used in this experiment were found to be equivalent to 1.03, 1.38, 1.56 and 2.39 ml. of 0.1 *N* KOH, respectively.

The results obtained with CoCl_2 are presented in Fig. 5. Maximum activation in the presence of cysteine was obtained only in buffered reaction mixtures, using a high Co^{++} concentration (0.006 *N*). In the absence of buffer, this maximum activation was not reached with any cobalt concentration. Co^{++} also produced activation in the absence of cysteine, the effect increasing as the ion concentration was increased. This effect was smaller, however, than that obtained in the presence of cysteine. The initial, cysteine, Fe^{++} and cysteine- Fe^{++} activities of the enzyme solution used in this experiment were 2.00, 2.75, 2.14 and 3.52 ml. 0.1 *N* KOH respectively.

Effect of O_2 and H_2 plus palladium, iodoacetic acid or hydrogen peroxide

10 mg. of palladium black were added to 4 ml. of enzyme solution. Hydrogen was passed through the mixture for 2 hr. at 37°. 1 ml. was then removed, without disturbing the palladium on the bottom of the reaction vessel, and tested for enzymic activity against gelatin by the usual 20 hr. anaerobic procedure (without addition of other activators). No effect on the initial activity was observed.

Other reaction mixtures were prepared by adding, to 1 ml. portions of enzyme, 5 ml. of buffer (*pH* 7), 3 ml. of 6.6% gelatin, 1 ml. of water and 10 mg. of palladium black. The mixtures were incubated for 20 hr. at 37° during which time they were continuously treated with O_2 and H_2 . Control experiments were run in the absence of palladium. A similar reaction mixture was incubated *in vacuo*. At the end of the experiment the $-\text{NH}_2$ increases which had occurred were measured as before. In agreement with previous results, the oxygen treatment had no effect on the enzymic activity, and the presence of palladium did not alter the result. The hydrogen treatment (absence of palladium) also had no effect; with hydrogen plus palladium a slight activation occurred. This may have been due to the metal catalyst alone, since a similar slight activation was obtained in the mixture incubated *in vacuo* with palladium.

Iodoacetic acid was tested by adding 10 and 20 mg. portions of the neutralized acid to mixtures containing 1 ml. of enzyme and the usual amounts of buffer and gelatin. Surprisingly, after 20 hr. anaerobic incubation, the $-\text{NH}_2$ increase was practically identical with that found in controls without iodoacetate. This substance very rapidly inactivates certain proteolytic enzymes such as cathepsin and papain, presumably by reacting with the active sulphydryl group of the enzyme molecule. The negative result obtained with the above bacterial proteinase indicates that its active group is not sulphydryl.

5 ml. samples of enzyme solution were mixed with 0.1, 0.5 and 1.0 ml. of 30% H_2O_2 , respectively. After 2 hr. incubation at 37°, the H_2O_2 was removed by dialysing for 1 hr. The initial (without activator) and full (cysteine- Fe^{++}) activities were then measured against gelatin on 1 ml. portions by the usual method. The lowest concentration of H_2O_2 had only a slight inhibiting effect on the activities. The two higher concentrations, however, had marked effects on both the initial and the full activities, the inhibition increasing with increasing H_2O_2 concentration. In other words, an irreversible inactivation occurs.

Table VIII. *Effects of O₂ and H₂ plus palladium, iodoacetic acid or H₂O₂*

Treatment	Activity (ml. 0.1 N KOH)
None (control)	3.35
Cysteine-Fe ⁺⁺ (control)	4.13
Oxygen (20 hr.)	3.35
Oxygen + palladium (20 hr.)	3.39
Hydrogen (20 hr.)	3.24
Hydrogen + palladium (20 hr.)	3.71
<i>In vacuo</i> + palladium (20 hr.)	3.78
Iodoacetic acid (10 mg.)	3.26
Iodoacetic acid (20 mg.)	3.20
Hydrogen peroxide (0.1 ml.): Initial	3.20
Full	3.62
Hydrogen peroxide (0.5 ml.): Initial	0.62
Full	0.68
Hydrogen peroxide (1.0 ml.): Initial	0.31
Full	0.14

Activation in presence of substrates other than gelatin

In order to determine whether the activation behaviour described for *Cl. histolyticum* proteinase is specific for gelatin, or holds true also for other substrates, parallel activity experiments were run using casein, Witte peptone, clupein sulphate and egg albumin as substrates. As with gelatin, these were made up in 6.6 % solution and brought to pH 7. The conditions of the experiments were identical with those of previous similar ones, excepting that in the case of egg albumin, which is difficult to hydrolyse, 2 ml. of enzyme were used instead of 1 ml., and that the total volume of incubation mixture was 12 ml. As before, incubation was carried out at 37° for 20 hr. in a hydrogen atmosphere. It was found that the Van Slyke method could not be used to estimate —NH₂ increase in the presence of casein because of precipitation. Therefore the Willstätter-Waldschmidt-Leitz alcoholic titration [1921] was used in this case; because of the disturbing effect of phosphate ions on this titration, the citrate-phosphate buffer had to be replaced by an equal volume of water. The pH did not change appreciably under these conditions. As shown in Table IX, the activation

Table IX. *Effect of various substrates on activation of Cl. histolyticum proteinase*

Substrate	Activities (ml. of 0.1 N KOH)			
	Initial	Cysteine	Fe ⁺⁺	Cysteine-Fe ⁺⁺
Gelatin	2.15	2.78	2.34	3.25
Casein	0.60	1.05	0.55	4.40
Witte peptone	0.48	0.85	0.86	1.26
Clupein sulphate	0.38	3.29	0.17	4.36
Egg albumin	0.27	0.32	0.0	1.01

behaviour is independent of the type of substrate used. In each case cysteine alone activates, Fe⁺⁺ alone is rather indifferent, whilst the cysteine-Fe⁺⁺ complex activates most strongly.

Activation of Clostridium histolyticum proteinase obtained from various culture media

Bacterial filtrates were prepared by inoculating sterile 4 % solutions of gelatin, casein, egg albumin and "Neopeptone" (Difco) at pH 7 with *Cl. histolyticum*, incubating for 3 days at 37° in a hydrogen atmosphere and filtering

through Berkefeld filters. 2 ml. portions were tested for proteinase activity against gelatin as previously described, using cysteine and Fe^{++} as activators. As indicated in Table X, the activation behaviour of the proteinase is independent of the nature of the culture medium upon which the micro-organism was grown.

Table X. *Activation of Cl. histolyticum proteinase obtained from various culture media*

Culture medium	Activities (ml. of 0.1 N KOH)			
	Initial	Cysteine	Fe^{++}	Cysteine- Fe^{++}
Gelatin	1.54	1.26	1.45	4.39
Casein	0.65	1.23	1.02	2.12
Egg albumin	0.18	0.27	0.20	0.98
"Neopeptone"	2.06	2.54	2.37	3.46
Horse meat broth	1.58	2.15	2.72	4.32

Rate of proteinase and aminopolypeptidase production by Clostridium histolyticum

300 ml. of horse meat broth were inoculated with *Cl. histolyticum* and incubated anaerobically at 37° . The number of bacteria was estimated at 24 hr. intervals by the method of Breed & Brew [1916]. Two samples were removed at the same time and tested for proteinase and aminopolypeptidase activity, (a) directly without filtration, and (b) after filtration through Berkefeld filters to remove bacteria. The proteinase activity was measured against gelatin on 1 ml. portions according to the usual method, using cysteine, FeSO_4 , or both, as activators. Aminopolypeptidase activity was measured by adding to 2 ml. of bacterial culture medium or filtrate 0.244 g. of *DL*-leucylglycine, 2 ml. of *N* ammonium chloride-ammonium hydroxide buffer (*pH* 9.5), and water to make a total volume of 10 ml. The *pH* of the mixture was 7.8; the incubation was carried out for 20 hr. at 37° . The liberated amino-groups were measured by the alcoholic titration method. Toluene was used as antiseptic during the incubation period involved in all enzyme determinations.

From Fig. 6 it can be seen that practically identical enzymic activities are exhibited by the unfiltered and filtered (bacteria-free) samples of the culture medium. The proteinase activity reaches a maximum at about 24 hr., after which it declines sharply. This behaviour strikingly corresponds with the number of bacteria present at corresponding times. (Bacterial counts indicated in Fig. 6, in thousands per ml., were as follows: 24 hr., 835; 48 hr., 625; 72 hr., 550; 96 hr., 200; 120 hr., 200; and 144 hr., 175.) Further study will be needed to determine whether the rapid decline in proteinase activity is due to its natural lability or to destruction by the bacteria themselves. It may be concluded, however, that most of the proteinase is secreted into the surrounding medium by the living bacteria, rather than liberated during autolysis. A maximum in the proteinase activity at 24 hr. was observed also when the activator was cysteine alone, or FeSO_4 alone.

A different picture is obtained for aminopolypeptidase. As shown in Fig. 6, the activity of this enzyme does not reach a maximum at 24 hr., but continues to increase slowly for 6 days. It was at first believed that this indicated a liberation of the enzyme by autolysis. However, the results shown in Table XI do not support this view. 200 ml. of horse meat broth were inoculated with *Cl. histolyticum* and incubated anaerobically for 24 hr. at 37° . The heavy growth of bacteria was centrifuged off, washed twice with saline solution, suspended in

17 ml. of water and incubated at 37° under toluene. At intervals, 2 ml. of suspension were removed and tested for aminopolypeptidase activity as previously described. As indicated, there was no increase in enzymic activity

Fig. 6.

- Proteinase activity of the bacterial suspension with cysteine + Fe⁺⁺
- ×—× Proteinase activity of the bacterial filtrate with cysteine + Fe⁺⁺
- Proteinase activity of the bacterial suspension without activator.
- ×---× Proteinase activity of the bacterial filtrate without activator.
- Aminopolypeptidase activity of the bacterial suspension.
- ×---× Aminopolypeptidase activity of the bacterial filtrate.
- △—△ Relative number of living bacteria ($\times 10^{-3}$ /ml.).

with increase in time of autolysis. It is likely that this enzyme, too, is secreted by the living bacteria. Perhaps after most of the high-molecular proteins have been decomposed by the proteinase, the remaining bacteria adapt themselves to the changed medium by secreting aminopolypeptidase, which can hydrolyse the protein breakdown products.

Table XI. *Aminopolypeptidase activity and autolysis of Cl. histolyticum*

Autolysis time (days)	0	1	3	5
Enzymic activity (ml. 0.1 N KOH)	0.15	0.10	0.10	0.10

SUMMARY.

1. The proteinase of *Clostridium histolyticum*, an organism belonging to the group of sporulating anaerobes, shows its maximum activity at pH 7, both in the presence and absence of added activators.

2. The proteinase is activated by sulphhydryl compounds, but not by the tryptic activator, enterokinase.

3. The activation produced by sulphhydryl compounds can be considerably increased by Fe⁺⁺, Mn⁺⁺, Ni⁺⁺, Cu⁺⁺ and Co⁺⁺. All except Co⁺⁺ produce activation at very low metal ion concentrations. The maximum activation is obtained under anaerobic conditions.

4. The active group in the enzyme is probably not —SH, since iodoacetic acid does not inhibit its activity. H₂O₂ in high concentrations inhibits irreversibly.

5. The quantity of proteinase present in a culture medium increases rapidly to a maximum during the early stages of bacterial growth (24 hr.), then decreases to a low value. The quantity of aminopolypeptidase increases steadily throughout the incubation period. These enzymes are probably secreted directly by the living bacteria.

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Addendum (19 July, 1937). Bacteria-free filtrates of *Cl. sporogenes* and *Cl. welchii*, which are, like *Cl. histolyticum*, strict anaerobes, have been found to behave, in all characteristics investigated, like *Cl. histolyticum*.