The Action of Proteolytic Enzymes on *Clostridium botulinum* type A Toxin

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Clostridium botulinum toxin is one of the most toxic substances known, the type A toxin containing about $220–250 \times 10^6$ mouse LD_{50}/mg. N (Putnam, Lamanna & Sharp, 1948). It is a protein of molecular weight about one million whose amino acid composition (Buehler, Schantz & Lamanna, 1947) as yet offers no indication of its mode of action.

As early as 1923, Schubel noted that the toxicity of culture filtrates of the organism was more sensitive to alkali than to acid and also was unaffected by pepsin and trypsin. Such observations have been confirmed repeatedly by other workers (Bronfenbrenner & Schlesinger, 1924; Nelson, 1927; Snipe & Sommer, 1928; Chistyakov & Rodopulo, 1943; Littauer, 1951), whilst Kalmanson & Bronfenbrenner (1943) reported that papain caused no destruction of the toxin.

These observations were concerned with crude toxin preparations but even with the crystalline material Abrams, Kegeles & Hottle (1946), Putnam *et al.* (1948) and Lamanna, Eklund & McElroy (1946) found that the protein is rapidly destroyed above pH 7, whilst Putnam *et al.* (1948) also recorded that 'even mild physical treatment of pure toxin produced loss of activity, particularly at high dilutions'.

In the present investigation the stability of partially pure and crystalline toxins has been examined at various pH values. The action of the crystalline enzymes, pepsin, trypsin and papain on the pure toxin has also been studied.

EXPERIMENTAL

Materials

Cl. botulinum *toxin.* Sarles & Herbert (1943, unpublished) reported that when cultures were allowed to stand at room temperature, after the usual period of incubation, much of the toxin was adsorbed on the fine precipitate of cell debris which settled out. The toxin can be recovered from the centrifuged deposit by extraction with an acid citrate buffer containing 0·18M sodium dihydrogen citrate, 0·02M disodium hydrogen citrate and M-NaCl, giving a pH of 4·0 (Bottomley & Creeth, private communication). The details whereby the toxin can be finally brought to the crystalline stage involve $(NH_4)_2SO_4$ fractionations after Lamanna, Eklund & McElroy (1946). All toxin specimens were prepared by extraction of the lysed culture precipitate, but whereas the first samples were crystalline their activities (about 70×10^6 LD_{50}/mg. N) were only about 30% of that quoted by Putnam *et al.* (1948), though later batches provided toxin with activity (250×10^6 LD_{50}/mg. N) comparable to that of the material isolated in the American laboratories (Putnam *et al.* 1948).

The two forms of crystalline and electrophoretically homogeneous toxin described above are hereafter termed 'partially pure' (low activity) and 'pure' toxin (high activity). Samples of both forms were used for investigating the stability of the toxin but only 'pure' toxin samples were employed in the enzymic work.

Enzyme preparations. Crystalline pepsin and trypsin were purchased (Armour Biochemicals) and their activities were determined according to Northrop, Kunitz & Herriot (1948) with haemoglobin. Crystalline papain was prepared and assayed according to Balls & Lineweaver (1939).

Methods

Non-protein nitrogen (N.P.N.). Sodium trichloroacetate, prepared by addition of alkali to the free acid to pH 2·0, was added to a sample of enzymic digest to give a final concentration of 0·25N trichloroacetate pH 2·0, under which conditions precipitation of toxin occurred rapidly. Free trichloroacetic acid could not be used because it inactivated the toxin. The sample was then centrifuged for 10 min. and subsequently filtered, or alternatively it was allowed to stand 0·5 hr. at 0° (low temperature to preserve toxicity) before filtration (Whatman no. 3 paper). As both procedures gave similar values for N.P.N. in the filtrate, the second method was finally adopted in view of its convenience. The filtrate was then adjusted to pH 6·0 and stored at 0° until assayed for toxicity.

Total nitrogen. Digestion for 4 hr. with 1 ml. 18N-H_2SO_4 containing 2·5 mg. $CuSO_4$, $5H_2O$, 20 mg. K_2SO_4 and 2 mg. Na_2SeO_4 was necessary in order to obtain 100% recoveries with not more than 70 μg. N. All reagents were of microanalytical reagent standard except the Na_2SeO_4 (N-free grade). Distillations were performed in the Markham (1942) apparatus and titrated with barium hydroxide from a Conway microburette.

Ninhydrin values. Owing to the small quantities of toxin available the spectrophotometric method of Moore & Stein (1948) was employed, taking the total unfractionated digest at pH 5·0, with a heating time of 30 min.

Toxicity determinations. Samples for assay were diluted in gelatin–phosphate buffer (Lamanna & Glassman, 1947) and 0·5 ml. was injected intraperitoneally into male albino mice of body weight about 20 g., using an average of nine animals for each dilution. The responses were examined by probit analysis and potencies expressed as LD_{50}/ml.

pH *determinations.* All readings were made with the glass electrode and rechecked at the end of the experiments. Unless otherwise stated citrate–phosphate buffer (McIlvaine) was used to obtain the required pH values.

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General procedure. The section of the work employing 'pure' toxin and crystalline enzymes involved the use of about 100 μg. toxin N/ml. and of 2–4 μg. enzyme N/ml. in citrate–phosphate buffer unless otherwise stated. At intervals, samples were removed into trichloroacetate (for N.P.N. estimation) and also into cold buffer solution, at a suitable pH to inhibit enzymic activity, e.g. for pepsin pH 6·0 and for trypsin pH 5·0. Hoover & Kokes (1947) have shown that papain is active over a wide range of pH values and hence, in the present investigation the papain–toxin solutions were adjusted to an arbitrary pH value of 5·0. All samples were diluted with cold buffer and immediately stored at 0° until assayed for toxicity. These solutions were analysed also for total N to confirm the accuracy of recovery at these stages.

RESULTS

The stability of the toxin in citrate–phosphate buffers at various pH *values*

Samples of the two forms of crystalline and electrophoretically homogeneous toxin described above, i.e. 'partially pure' (low activity) and 'pure' toxin (high activity), not only exhibited differences in activity but also in stability under various pH conditions.

A sample of 'partially pure' toxin solution (17×10^6 LD_{50}/ml.) in 3·5N-HCl at 16·2° retained only 1 % of its original activity after 2 min. In 5 hr. the remaining toxicity had also disappeared but no more N.P.N. was released than from the original untreated toxin solution after precipitation directly with trichloroacetate. Solutions of both forms of toxin (2×10^6 LD_{50}/ml.) appeared to be stable at 0° in citrate–phosphate buffer; the 'partially pure' material at pH 6·5 exhibited no loss of toxicity in 8 days whilst a similar retention of activity was shown by the 'pure' toxin at pH 5·0 for at least 40 days. On the other hand, both forms of toxin at this concentration lost all activity after 20 min. at 99°.

The stability of the 'partially pure' material was examined at the same concentration (2×10^6 LD_{50}/ml.) in citrate buffer pH 7·0 and 30° as a preliminary to the use of trypsin; the results in Fig. 1 show rapid loss of activity.

'Pure toxin' (2×10^6 LD_{50}/ml.) was similarly examined for stability. At pH 7·0 and 25° and pH 7·0 and 30·0° no loss was detected up to 26 hr., or at pH 7·0 and 38° in 2 hr. Further information on the greatly increased stability of the 'pure' toxin is given by the control experiments conducted with trypsin at pH's 6·6 and 7·6 (see later).

Action of proteolytic enzymes on the 'pure' toxin

Pepsin. The toxin, adjusted to pH 2·0 with dilute HCl and containing crystalline pepsin (2 % of toxin on N basis), was incubated at 29·6° for the periods shown in Fig. 2.

A probit analysis of the unfractionated solutions indicated that the LD_{50} was very similar for all control (toxin and buffer only) solutions and for the toxin solution containing enzyme tested immediately after mixing. Samples of toxin incubated with the enzyme showed a significant fall in toxicity within 3 hr., the level then remaining virtually constant up to 21 hr. Statistical analysis of the results of the toxicity determinations indicated that the difference between the mean values of the untreated and combined pepsin-treated (3–21 hr.) toxins were significantly different at the 1 % level and demonstrated a loss of about 25 % toxicity in presence of pepsin. The N.P.N. fractions of both control and experimental solutions had no toxicity.

The N.P.N. in the control remained steady at a small value during the incubation. On the other hand, in the peptic digest the N.P.N. increased rapidly and followed closely the loss of toxicity in this period. Therefore the initial value for N.P.N. in the peptic digest of Fig. 2 was calculated from the control solution. The identical values obtained for total N in the first samples for control and experimental solutions show that little error could have resulted from this assumption.

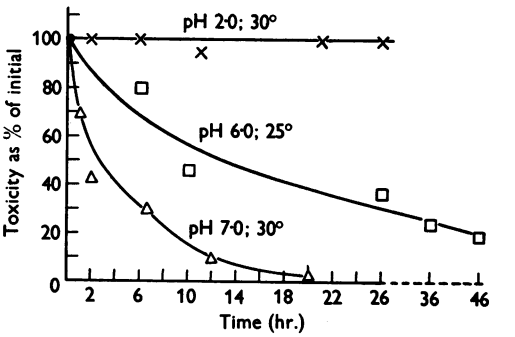

Fig. 1. Effect of pH and temperature on 'partially pure' toxin.

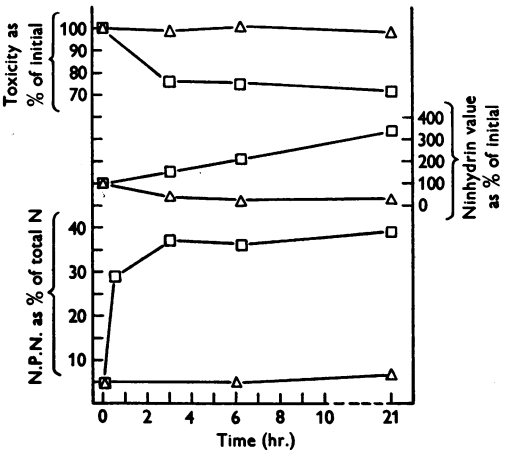

Fig. 2. Effect of pepsin on 'pure' toxin at pH 2·0 and 30°. △, toxin only; □, toxin and enzyme.

Trichloroacetate fractionation provides only limited information on the possible action of enzymes on proteins as degradation to molecules still large enough to be insoluble in trichloroacetate may have occurred. The course of the reaction was therefore also followed with the ninhydrin method because of its suitability for the small amounts of toxin concerned. Fig. 2 indicates an increase in ninhydrin value in the early stages of digestion at the same time as the N.P.N. is increasing. However, the extent of the rise in ninhydrin value suggests that the products formed in the N.P.N. fraction are not small peptides (cf. Moore & Stein, 1948; Dowmont & Fruton, 1952). Even when the N.P.N. reaches its maximum and constant level the ninhydrin value is still increasing.

No explanation is available for the decrease in ninhydrin-positive material in the control which had been kept at pH 2·0 and then brought to pH 6. However, it is known that changes in the dissociation of *Cl. botulinum* toxin occur in solution between pH 2·0 and 4·3 (Wagman & Bateman, 1951).

Trypsin. Previous reports had indicated that crystalline toxin was more stable on the acid side of pH 7·0; therefore in the present work with 'pure' toxin the action of trypsin (4 % of toxin on N basis) was first investigated in citrate–phosphate at pH 6·6 and 25·5°. Although this pH is below the accepted pH optimum for the enzyme the activity was found to be about 50 % of that at pH 7·6 when determined according to Northrop *et al.* (1948) with haemoglobin as substrate.

Fig. 3 illustrates that the 'pure' toxin itself is stable under these conditions, no significant loss of activity occurring in 26 hr., whereas in the presence of trypsin rapid loss of activity ensues in 3 hr. followed by a slower but progressive loss of up to about 90 % activity in 26 hr. The N.P.N. fractions, both control and experimental, exhibited no toxic activity at any stage.

The N.P.N. in the control samples like those in the peptic incubation had a low and constant level during the incubation. In the presence of trypsin, however, a slow and progressive increase in N.P.N. began at the end of the rapid phase of inactivation of the toxin.

In the absence of trypsin the ninhydrin determinations indicated a slight continual increase whilst in the experimental digest little or no alteration was seen during the rapid detoxication phase. Subsequently, a sharp rise in ninhydrin value ensued coincident with both a slight inactivation of toxin and with rise in N.P.N. and was followed by a steady decline to slightly below that of the control (see Discussion).

After it had been shown that the 'pure' toxin was stable at about pH 7·0 and higher, the action of trypsin at pH 7·6 and 25·5° was examined. Fig. 4 shows that no loss of activity was apparent at this pH unless trypsin was present in which case about 90 % loss of toxicity occurred as at pH 6·6.

The ninhydrin results in this experiment also closely follow those at pH 6·6. Very little change was found in absence of trypsin and even with enzyme present no great alteration in ninhydrin value was apparent when toxicity was decreasing rapidly. Later, however, a marked increase in ninhydrin-positive material was found simultaneously with a phase of less rapid but significant

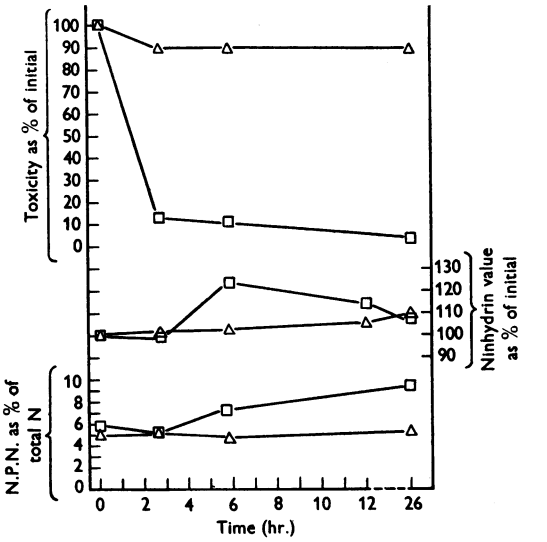

Fig. 3. Effect of trypsin on 'pure' toxin at pH 6·6 and 25°. △, toxin only; □, toxin and enzyme.

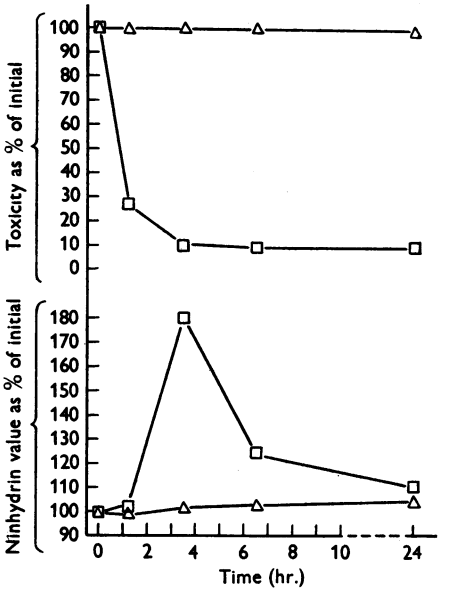

Fig. 4. Effect of trypsin on 'pure' toxin at pH 7·6 and 25°. △, toxin only; □, toxin and enzyme.

detoxication. After this point the toxicity decreased only slightly (cf. Fig. 3) whilst the ninhydrin values gradually diminished (cf. Fig. 3).

Papain. The crystalline enzyme was activated with half its weight of cysteine for 1 hr. at pH 7·0 before mixing (4 % on basis of total N present) with toxin in citrate–phosphate buffer of pH 4·9 at 25·3°.

Fig. 5 indicates that no loss of activity occurred at this pH either in the presence or absence of papain and no toxic activity appeared in the N.P.N. fraction. Likewise, the N.P.N. itself remained constant and of approximately the same value in control and experimental solutions during the whole incubation.

In the absence of papain, the ninhydrin value altered little throughout the experiment, but in the presence of the enzyme an increase was observed in the early stages (in triplicate observations) followed by a fall to a relatively constant value.

DISCUSSION

The rapid inactivation of the 'partially pure' toxin at about pH 7 and its stability at 0° and in acid solutions (pH 2·0) is in agreement with work reported from other laboratories using crude or crystalline preparations of toxin.

The stability of the 'pure' toxin at about pH 7 is contrary to work reported elsewhere either with crude (Littauer, 1951) or crystalline (Abrams *et al.* 1946; Putnam *et al.* 1948) toxins. The toxin employed by the latter groups of workers corresponded somewhat in stability to that of the 'partially pure' toxin used here, which was rapidly inactivated at pH 7.

Examination of the effect of the three enzymes on the toxin shows that papain has no inactivating action, pepsin produces a slight detoxication, whereas trypsin effects almost complete inactivation. The result with papain is similar to that reported elsewhere with crude preparations of toxin (Kalmanson & Bronfenbrenner, 1943), whereas the slight initial loss of activity that occurs with pepsin seen here was not apparent with the impure toxins used by earlier investigators. Likewise, previous reports on the lack of action of trypsin on unpurified preparations of toxin could not be confirmed when crystalline enzyme and 'pure' toxin were employed. Such differences may perhaps be due to the presence of protective impurities in some impure samples of toxin.

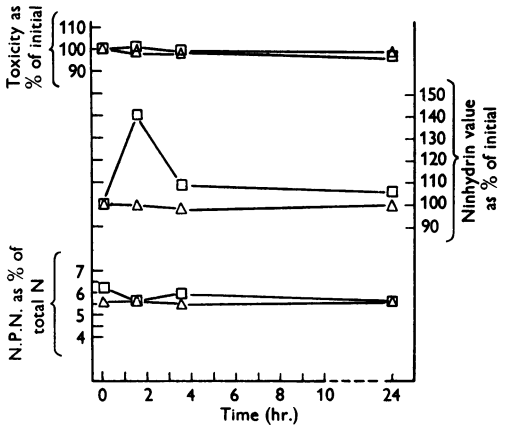

Fig. 5. Effect of papain on 'pure' toxin at pH 4·9 and 25°. △, toxin only; □, toxin and enzyme.

In none of the enzymic experiments with papain, pepsin and trypsin could toxicity be detected in the soluble N.P.N. fraction. Only in the case of the peptic digest did the N.P.N. rise much higher than in the control without enzyme. In view of the evidence of Lamanna & Lowenthal (1951) that type A toxin consists of two components—a haemagglutin and a true toxin—it is possible that in the present work pepsin is primarily attacking the haemagglutin rather than the toxic factor. By this means a large increase in N.P.N. would be achieved with a possible slight loss in toxicity.

The ninhydrin values in the experiments with trypsin and papain are of particular interest in that with the former enzyme the ninhydrin value, like the N.P.N., remains relatively unchanged whilst the most rapid detoxication is occurring. With papain a slight increase in ninhydrin value takes place although no toxic activity is lost. Such observations suggest that loss of toxicity can ensue from minor modification of the original protein and not necessarily by gross degradation of the molecule.

Note added in Proof. Since this work was completed I. W. Coleman (*Canad. J. Biochem. Physiol.* (1954) **32**, 27) has reported the action of pepsin and trypsin on the toxicity of crystalline toxin. In both cases, however, the inactivation was very much smaller than in our experiments, whilst the toxin itself corresponded in purity and stability to our 'impure' material.

SUMMARY

1. Two forms of crystalline *Clostridium botulinum* type A toxin have been prepared. One of these was low in specific activity ('partially pure') and was highly unstable at pH 7·0, whilst the second form possessed much higher specific activity ('pure' toxin) and was more stable at pH 7·0.

2. The action of crystalline proteolytic enzymes on the 'pure' toxin has been investigated. With the conditions adopted, papain had no inactivating effect, pepsin produced slight detoxication whilst trypsin greatly lowered the toxicity. In none of the enzymic experiments was any toxic product of low molecular weight produced.

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The Antithrombin Activity of Human Plasma

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The presence of an agent in serum capable of progressively inactivating the thrombin formed during the coagulation of blood or plasma has long been recognized. After it had been suggested that the factor involved in this action was protein in nature (Rettger, 1909), three main ideas were put forward as to the method of inactivation. Landsberg (1913) considered that thrombin was inactivated by a reversible non-specific adsorption on to serum albumin; Collingwood & MacMahon (1912) held that thrombin was destroyed by a specific enzyme in serum; and Howell (1916) and his co-workers maintained that thrombin was rendered inactive by combination with a specific protein, the product of this combination being termed metathrombin.

More recent work has shown that antithrombin occurs in the albumin fraction of serum as obtained by salt precipitation (Lenggenhager, 1935; Quick, 1938; Stewart & Rourke, 1940; Astrup & Darling, 1942), but the factor itself has not been isolated. Astrup & Darling (1942), working with bovine materials, described a method for the quantitative estimation of antithrombin based on the fact that a given quantity is capable of inactivating only a definite number of thrombin units, and that this inactivation is complete in 15 min. Several observations recorded in the literature indicated that this method of measurement might not be directly applicable to the estimation of human antithrombin. Both Collingwood & MacMahon (1913) and Quick (1938), working with human materials, observed that thrombin inactivation continues for at least 30 min., while Herbert (1940) found that in a given time human serum would inactivate a definite fraction of the thrombin present, independent of its absolute concentration.

These apparent differences between the behaviour of human and bovine antithrombin indicated that a more complete examination of the reaction between human thrombin and human antithrombin might yield new information.

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MATERIALS AND METHODS

Veronal buffer. Unless the contrary is stated, all the dilutions of materials used were carried out with a veronal buffer (Michaelis, 1930) of pH 7·3, $I = 0{\cdot}15$. Two litres of this buffer contained 215 ml. 0·2N-HCl, 14·43 g. NaCl, and 11·74 g. Na barbiturate.

Fibrinogen. The estimation of antithrombin depends on measuring the inactivation of thrombin, and the strength of thrombin is measurable only in terms of the rate of coagulation of fibrinogen, whether this be native fibrinogen in plasma or a solution of fibrinogen purified by fractionation. In these experiments a solution of bovine fibrinogen was used as coagulation substrate, the fibrinogen being obtained by precipitation from bovine plasma by addition of 0·11 vol. ether at 0°. The precipitate was washed with citrate saline containing 8% (v/v) ether, redissolved in citrate saline, and freeze-dried. The fibrinogen obtained in this way appears to be electrophoretically and ultracentrifugally homogeneous. It gives a satisfactory solution for measuring thrombin