THE EXTRACELLULAR PROTEOLYTIC SYSTEM OF CLOSTRIDIUM PARABOTULINUM

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The enzyme complements of the proteolytic anaerobic bacteria have been widely investigated, but strangely enough *Clostridium botulinum* has been neglected in this respect. In view of the importance of this organism both theoretically and practically, as a producer of a potent neurotoxin, we have attempted to determine the nature and properties of the various enzymes which catalyze proteolysis by this organism and the relation of these enzymes to toxin production.

Wagner, Meyer and Dozier (1925) stated that the gelatinase which was produced in cultures of *C. botulinum* was influenced by the carbohydrate concentration of the medium and they observed a more rapid disappearance of the gelatinase in the presence of carbohydrate than in its absence.

Snipe and Sommer (1928) in their work on purification of botulinus toxin found that a gelatinase, a peptidase, and a lipase were precipitated with the toxin by acidification of the supernatant liquid from cultures.

Maschmann (1927, 1938) has also made a study of the proteolytic enzymes of *C. botulinum*. He found that "there is a proteinase, an aminopolypeptidase and a dipeptidase secreted in the medium during growth." These enzymes were determined by the amino-group titration procedure. They were activated by M/250 cysteine, M/100 hydrogen cyanide and were unaffected by iodoacetic acid. Maschmann also found that cysteine was necessary before the proteinase would attack clupeine.

The objections, however, to using amino group or carboxyl group determinations for estimating proteinase activity have been clearly stated by Northrop (1932) and Anson (1932). "The splitting of peptide linkages which is what is measured by determination of the number of amino and carboxyl groups freed is due not only to proteinases but also to peptidases which cannot digest proteins. The estimation of the proteinases of anaerobes by Maschmann are thus estimations not of proteinase alone but of proteinases plus peptidases." (Anson, 1938).

EXPERIMENTAL

Northrop (1932) determined that the first effect of proteinases such as pepsin and trypsin on proteins (gelatin, casein and edestin) was to cause a rapid decrease in the viscosity of the protein. A measure of the viscosity changes during the first few minutes of contact between "enzyme solution" (culture supernatant) and protein will therefore give an accurate estimate of the true proteinase concentration.

The gelatin and casein solutions were prepared as recommended by Northrop (1932) for standard methods of peptic digestion. The actual viscosimetric determination of proteinase also followed Northrop's procedure (1932) with minor alterations.

The equation which was used to evaluate culture fluids for proteinase in this method was that suggested by Northrop (1932):

$$N = \frac{ts}{t\mathrm{H_2O}} - 1$$

where N = the specific viscosity of the protein solution, ts = time of outflow of protein solution, $t\mathrm{H_2O}$ = time of outflow of water from same viscosimeter.

The values of N were then plotted against the time elapsed after the enzymes and substrate solutions were mixed. The percentage change in viscosity for a given period was then interpolated from the curves. The zero point was determined by extrapolating on the curve obtained with a portion of enzyme solution previously boiled.

The per cent changes obtained by interpolation for a group of dilutions were averaged and the mean value determined for a digestion period of thirty minutes. It will be seen from the data below that the per cent change caused by different dilutions of culture fluid varied directly with the reciprocal of the dilution used. The error determined with a time period of thirty minutes was within 5 per cent. When the period was taken at twenty minutes the error was consistently larger and therefore thirty minutes was chosen as the uniform point of calculation. (See fig. 1.)

It is obvious from the data that the method allows definite appraisal of the proteinase content of a supernatant fluid.

TABLE 1

Per cent change caused in viscosity of 2.5 per cent gelatin solution, pH 7.0 by various amounts of proteinase of C. botulinum

Temperature of reaction 35.5° C.

DIGESTION TIME	PER CENT CHANGE IN VISCOSITY				VALUE OF K				MEAN VALUE OF K	DILUTION RECIPROCAL CALCULATED MEAN K PER CENT CHANGE			
	Dilution reciprocal												
	2	3	4	5	2	3	4	5		2	3	4	5
minutes													
20	55.3	37.5	25.8	21.4	110.6	112.5	113.2	107	110.8	2.00	2.95	4.29	5.17
30	67.6	44.6	33	26.7	235.2	133.8	132	133.5	133.6	1.97	2.99	4.04	5.00

However there are certain points limiting the interpretation of any viscosity change of a protein by such an "enzyme solution." The most important is that by this method we have probably measured only one type of proteinase, "gelatinase," and have not measured the total proteinase activity. Other proteinases such as that responsible for milk clotting should also be investigated, not only to determine their presence but also to check the validity of the viscosimetric method. The investigation of the milk-clotting power of culture fluids of *C. parabotulinum* on purified enzyme preparations will form the subject of a subsequent paper.

It was next imperative that certain properties of the proteinase

be defined in order to determine the set of conditions under which it could be routinely evaluated in the future and, if possible, its relation to other proteinases.

Experiment 1. Relation between proteinase activity and pH

Isoelectric gelatin was adjusted to the desired pH with HCl or NaOH. The supernatant fluids from forty-eight-hour-old cultures were collected and brought to the proper pH value

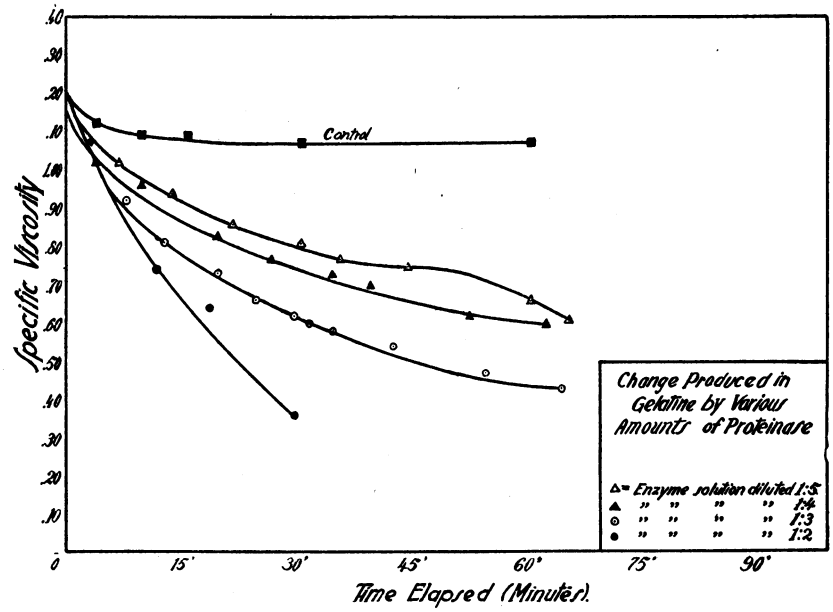

FIG. 1

before addition to the substrate. All pH values were controlled electrometrically.

The greatest change in the viscosity of a gelatin solution was obtained in thirty minutes at pH 7.0. In this respect the proteinase of *C. botulinum* differs from the animal proteinases, since the optimum pH for pepsin is about 2.5, for the cathepsins 3.7, for pancreatic proteinase 9, and for papain 7.4. On the other hand, the value obtained here agrees well with those for *Clostridium histolyticum*, *Clostridium sporogenes*, and *Clostridium*

welchii as reported by Weil and Kocholaty (1937), and other bacterial proteinases.

We did not at this time attempt to answer the question con-

TABLE 2

Summary of the pH-activity data

pH	PER CENT CHANGE IN 30 MINUTES
8.0	59
7.0	71.5
6.0	62.6
4.95	11.4
4.0	0
3.0	0

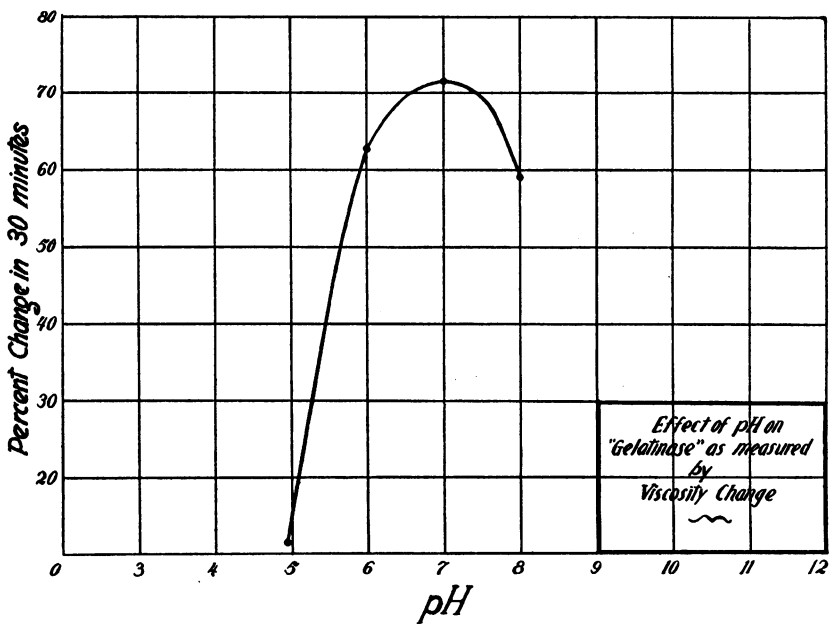

FIG. 2

cerning the effect of pH on the enzyme and on the actual viscosity change. It is quite possible that our results in this section do not represent an effect of pH on the activity of the enzyme but rather the effect of pH on the rupture of the linkages in the

protein molecule. In other words, the change in viscosity per bond hydrolyzed may be greatest at a given pH. We may have been measuring this latter effect.

Having determined the "pH of maximal activity," it remained to characterize the proteinase by activations and inactivations.

a. The effect of cysteine. Cysteine hydrochloride solution, neutralized before testing, was added to aliquots of proteinase preparations so that there were final concentrations of 0.082 M and 0.0082 M cysteine per cubic centimeter enzyme solution. The mixtures were placed in a vacuum desiccator and incubated *in vacuo* for thirty minutes. The proteinase solution was then removed and its activity against gelatin determined viscosimetrically. All components of the digestion mixture were ad-

TABLE 3

Effect of cysteine

PROTEINASE PREPARATION	PER CENT CHANGE IN VISCOSITY OF GELATIN SOLUTION
(a) Untreated preparation	64.2
(b) Heat-inactivated	0.0
(c) Treated with 0.0082 Mols cysteine	64.2
(d) Treated with 0.082 Mol cysteine	52.6

Inactivation by cysteine = 11.6 per cent.

justed to pH 7.0 electrometrically. Controls were composed of (*a*) suitably diluted active proteinase preparations and (*b*) boiled enzyme plus 0.082 M cysteine. The results are presented in table 3 and figure 3. Unlike the proteinase of *C. histolyticum* (Weil and Kocholaty, 1937) cysteine inhibited that of *C. botulinum*. This effect, plus the action of cyanide to be discussed subsequently, indicates that the proteinase of *C. botulinum* is not of the papainase type.

b. The effect of sodium cyanide. Solutions of sodium cyanide in M/15 phosphate buffer, pH 7.0, were prepared so that there were 0.2 mol and 0.02 mol cyanide salt per cubic centimeter. Equal volumes of proteinase preparation and cyanide solution were incubated at 37°C. for thirty minutes, after which aliquots

were removed to gelatin solution and tested viscosimetrically. Control tubes included (*a*) heat-inactivated enzyme solution containing 0.2 mol of sodium cyanide per cubic centimeter, (*b*)

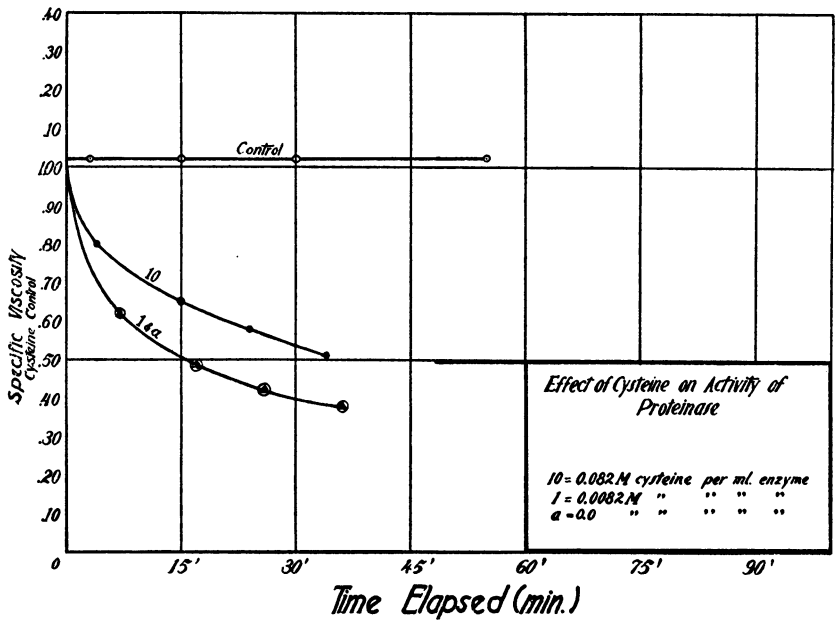

FIG. 3

TABLE 4

Effect of sodium cyanide on proteinase

PROTEINASE PREPARATION	PER CENT VISCOSITY CHANGE
(a) Untreated	45.3
(b) Heat Inactivated plus 0.1 Mol cyanide	0.0
(c) Heat Inactivated	0.0
(d) Treated with 0.1 Mol cyanide per cc. proteinase	0.0
(e) Treated with 0.01 Mol cyanide per cc. proteinase	45.3

Inactivation by 0.1 Mol sodium cyanide = 100 per cent.

heat-inactivated enzyme suitably diluted with distilled water, and (*c*) untreated enzyme suitably diluted. All pH values were checked before the mixtures were placed in the viscosimeter.

A total inhibition of the proteinase preparation by 0.1 mol sodium cyanide was observed, at pH 7.0.

Altering the pH to 5 and 9 during the cyanide inactivation did not change the effect, as Lawrie (1937) had observed to be the case with the proteinase of *Glaucoma piriformis*. The results obtained in this work are also contradictory to those of Maschmann (1937) who based his proteinase determinations on amino

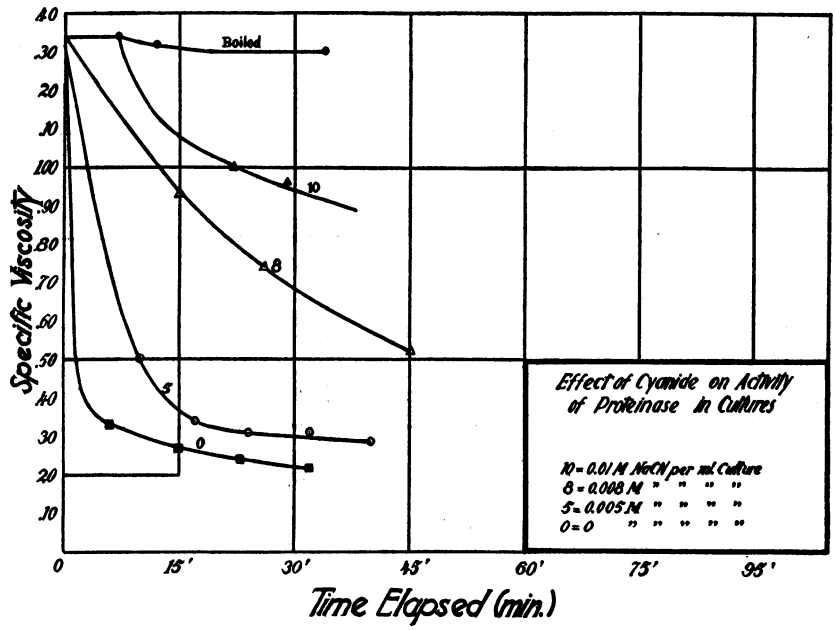

FIG. 4

group titrations. He found that M/100 hydrogen cyanide and M/250 cysteine activated the proteinase of *C. botulinum*.

Copper sulfate in the amount of 6×10^{-5} mol per cubic centimeter of proteinase also showed a complete inactivation viscosimetrically, both in air and *in vacuo*, in this series of experiments. The *in vacuo* tests were carried out to check the possible action of copper in catalyzing the uptake of oxygen or the oxidation of the proteinase.

It must be observed that the fundamental difference in the

methods which were used in these experiments and by Maschmann to determine "proteinase activity" may well be the cause for the divergence of results. The viscosimetric method is one predominantly involving a physical change in the micelle structure of the protein substrate. It certainly has been proven that amino nitrogen or carboxyl group titrations measure the entire proteolytic activity and not the action of the proteinase alone. Hence the seemingly contradictory results on inactivation phenomena are not strictly comparable.

c. The effect of hydrogen peroxide. To determine whether the active group on the proteinase of *C. botulinum* was of sulfhydryl nature, the action of hydrogen peroxide was tested. It was conceivable that the peroxide might oxidize such a group which

TABLE 5

Effect of hydrogen peroxide

PROTEINASE PREPARATION	PER CENT VISCOSITY CHANGE
Untreated, dialyzed	45
Heat Inactivated, dialyzed	0.0
Treated with 0.1 cc. peroxide	34.3
Treated with 1.0 cc. peroxide	31.2

Inactivation by peroxide = 13.8 per cent.

could be reactivated by the addition of hydrogen sulfide to reduce the action.

Five-cubic-centimeter aliquots of a 30-hour-old supernatant fluid at pH 7.0 received 0.1 and 1.0 cubic centimeter amounts of a 30 per cent hydrogen peroxide solution. The mixtures were incubated one hour at 37°C. and then dialyzed for one hour against distilled water in cellophane tubing to remove the peroxide. One-cubic-centimeter amounts were tested on gelatin solution. Control tubes checked the effect of (*a*) dialysis on the enzyme and (*b*) boiled dialyzed proteinase.

Attempts to reactivate the peroxide-treated proteinase solution by hydrogen sulfide and thioglycollic acid were made but were not successful. It appeared that the peroxide exerted an irreversible inactivating effect.

The ability to decrease the viscosity of gelatin solutions was paralleled by similar alterations in casein solution. However, in this respect quantitative differences were noted, namely in the amount of proteinase solution required to cause such a change. It was found that the concentration of the enzyme, considered as per cent of the total volume of the digestion mixture, had to be raised to 40 per cent for casein digestion, whereas with gelatin it amounted to 16 per cent.

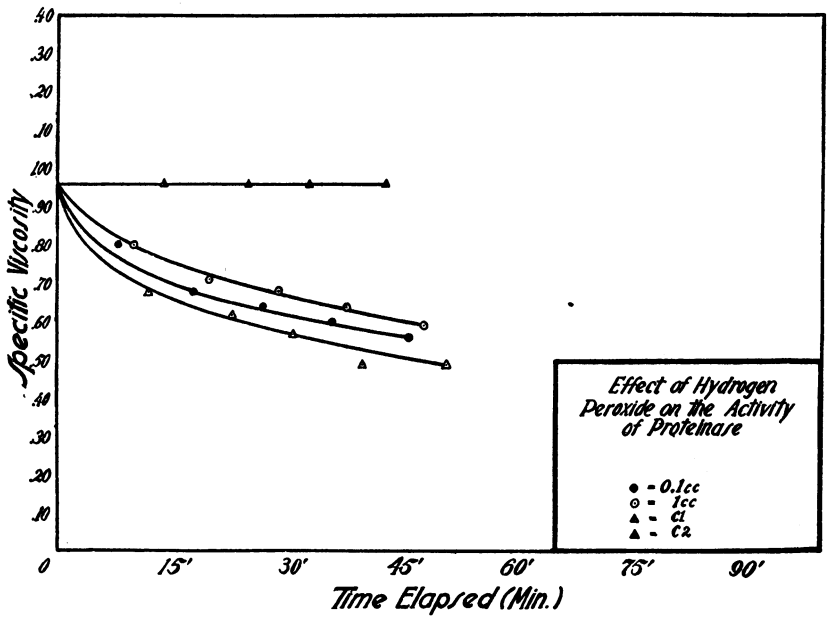

FIG. 5

The curves obtained with casein also lack the large initial change in per cent viscosity (0–15 minutes) so characteristic of gelatin digestion. A typical determination with casein is presented.

Three cubic centimeters of a 5 per cent casein solution at pH 7.0 plus 2 cc. of a proteinase preparation were tested in the usual manner. The control was composed of a boiled proteinase preparation.

It will be seen from the curves that the proteinase was able

to accomplish a 46.2 per cent change in the viscosity of the casein within thirty minutes.

Experiments on pH-activity relations, cysteine, cyanide and peroxide effects were carried out leading to the same results obtained with gelatin.

Edestin was also acted upon by the proteinase of *C. botulinum*. However, the results with this protein were not as clear cut as with gelatin or casein owing to the relative insolubility of the protein at the pH of optimum activity. Minor variations in the pH of the digestion mixture frequently caused minute flakes of edestin to form in the viscosimeter. As a result, most of the experiments on edestin had to be carried out around pH 8.0. The data at this point were however comparable to those for gelatin at the same pH.

To determine the extent of digestion of the protein of which the proteinase was capable, it was decided to use Willstätter and Waldschmidt-Leitz's alcoholic titration of the amino group (1921). Although such a method does not estimate proteinase alone, but the total activity of all the proteolytic enzymes in the fluid, it was felt at this time that preliminary experiments on whole proteins would pave the way in standardizing this procedure for the determinations of the lower peptidases.

> There are approximately three typical methods for estimating amino nitrogen. These are (*a*) the nitrous acid method of Van Slyke (*b*) the formaldehyde method, and (*c*) the alcoholimetric titration. None is entirely satisfactory. The Van Slyke nitrous acid method is tedious, fortuitous errors arise from frothing and reducing agents in solution, and it gives faulty values for several amino acids and peptides. [Richardson, 1934.]

The formaldehyde and alcohol titrations are rapid and were specifically developed for enzyme and protein chemistry. Furthermore, with a glass electrode available the end points are not difficult to attain, although they are on the alkaline side in a range where the electrode works with less precision. With control solutions treated identically, the values are only used in relation to each other and approximately the same electrode

error obtains in both the test and control series. The error of the electrode in this region (10.5) is equalled by the error in the colorimetric method.

TABLE 6

Alkalimetric determination of pH-activity curve

pH OF PROTEINASE PREPARATION	N/5 KOH	PER CENT CHANGE (BASED ON CHANGE AT pH 7.15)
	cc.	
5.0	0.2	36.3
6.0	0.32	58.1
7.15	0.55	(100.) (arbitrary)
8.0	0.23	41.8
9.15	0.19	34.5

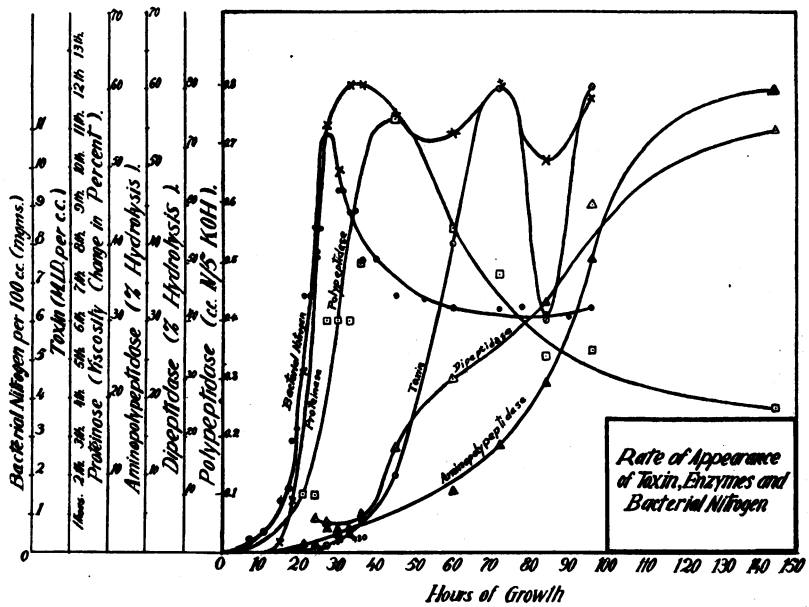

FIG. 6

The presence of phosphate buffer salts in the medium which interfered with the formol titration, plus the possible stepwise procedure in the alcoholic method, made it more profitable to use the latter method.

For completion the following experiment, relating activity to pH range, is presented.

Fifteen cubic centimeters of gelatin solution adjusted electrometrically with hydrochloric acid or sodium hydroxide to the desired pH were mixed with 3 cc. of supernatant fluid, also adjusted to proper pH value, and incubated 18 hours at 37°C. Controls consisted of boiled proteinase preparations or, in other cases, aliquots of the test mixture titrated immediately on mixing. The pH was adjusted to 7.0, and absolute alcohol added until the final alcohol concentration was 90 per cent. The mixtures were then titrated with N/5 KOH in 90 per cent alcohol to pH 10.5 by means of the glass electrode.

Comparison between the shape of the curve obtained by alkalimetric determination and that obtained viscosimetrically reveals an identity of optimum points but also shows in the former case a more gradual loss of activity on the alkaline side.

THE POLYPEPTIDASES

a. "Polypeptidase" activity was determined by using a 1 per cent Witte's peptone solution, at pH 7.4. Eight cubic centimeters of the peptone solution were mixed with 4 cc. of the supernatant fluid, and a 5 cc. aliquot withdrawn immediately for the control titration. The remainder of the digestion mixture was incubated at 37°C. and a 5 cc. aliquot titrated.

b. Aminopolypeptidase activity was determined with d,-l-leucylglycyl-glycine. A M/10 solution was prepared and adjusted to pH 7.8; 4 cc., containing 0.0004 mol, were mixed with 2 cc. supernatant fluid. A 3 cc. aliquot was removed immediately for the control titration and the remainder incubated at 37°C. for 18 hours.

c. Carboxypolypeptidase activity was determined with chloracetyl-l-tyrosine as substrate. A M/25 solution was used, of which 10 cc., containing 0.0004 mol, at pH 7.8, was mixed with 4 cc. supernatant fluid. An aliquot of 7 cc. was removed immediately for the control titration and the rest was incubated 18 hours at 37°C.

d. Dipeptidase was determined with d,l-leucyl-glycine and

glycyl-glycine as substrates. M/10 solutions were prepared, of which 4 cc., containing 0.0004 mol, at pH 8.0, were mixed with 2 cc. supernatant fluid. A 3 cc. aliquot was removed immediately for the control titration and the remainder titrated after 18 hours at 37°C.

e. Tri-glycine splitting ability was determined with diglycyl-glycine, using M/10 solution, at pH 7.8. The digestion mixture contained 4 cc. (0.0004 mol) substrate plus 2 cc. supernatant fluid. The usual control was titrated and the test incubated 18 hours at 37°C.

It was to be expected from the growth of *C. botulinum* in commercial peptones that a "polypeptidase" would be present in the

TABLE 7

Peptidase activity of a 48-hour-culture supernatant fluid

ENZYME DETERMINED	TITRATION VALUE (CC. N/5 KOH)	PER CENT OF THEORETICAL HYDROLYSIS
1. "Polypeptidase" (48 hrs.)	0	0
(24 hour culture)	0.8	
(72 hour culture)	0.25	
2. Aminopolypeptidase	0.03	3.0
3. Carboxypolypeptidase	0.0	0
4. Dipeptidase:		
(a) Leucyl glycine	0.06	6
(b) Glycyl glycine	0.0	0
5. Tripeptidase (Diglycyl glycine)	0.0	0

extracellular system. The significance of this hydrolysis is of course not clear, owing to the uncertainty of the chemical nature of the substrate, and to the fact that proteinases also attack peptones. The ability of the supernatant to decompose "Proteose Peptone" would indicate the ability of the organism to produce peptidases acting on polypeptides of more than three amino acids in the chain.

The lack of ability to decompose chloracetyl-l-tyrosine, indicating the absence of a carboxypolypeptidase, was confirmed by testing supernatant fluids every 24 hours over a period of 240 hours at pH values from 4 to 10. At no time was there any indication of such an enzyme in the extracellular system.

The presence of an aminopolypeptidase was confirmed repeatedly. It was also determined that after 5 to 6 days the hydrolysis would exceed 50 per cent, indicating that both optically active forms were hydrolysed. This enzyme in supernatant fluids of *C. botulinum* was also found by Maschmann and he observed that it was specifically inactivated by cysteine and cyanide.

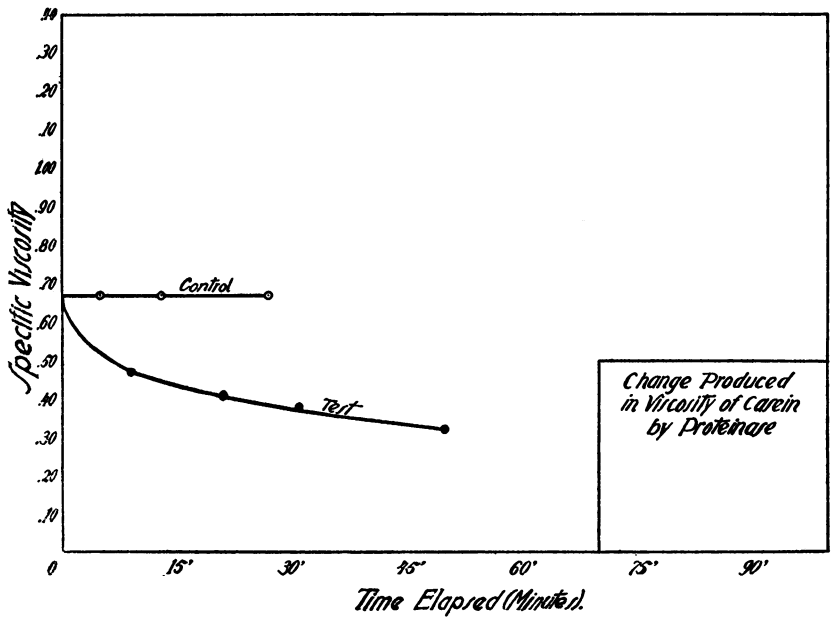

FIG. 7

The presence of a dipeptidase was confirmed, and in the case of *Clostridium parabotulinum*, it was also found that peptides containing only molecules of glycine were not hydrolysed. Johnson and Peterson (1935 a, b) noted this same specificity in the case of the dipeptidase of *Aspergillus parasiticus*. It was observed that, in the case of *C. botulinum*, apparently both optically active forms were hydrolysed inasmuch as the per cent hydrolysis was often equivalent to more than 50 per cent.

The inability to hydrolyse diglycyl glycine further emphasized the specificity of the polypeptidases.

The optimum points for activity of the aminopolypeptidase and dipeptidase in the extracellular system were determined to be pH 7.8 and 8.0 respectively.

It was decided to postpone indefinitely further experiments on activation and inactivation procedures on these enzymes until a time-correlation between them and toxin production could be tentatively established.

The extracellular proteolytic system of *C. parabotulinum*, A, apparently consists at least of a "proteinase," which changes the viscosity of gelatin, casein and edestin, and aminopolypeptidase, a dipeptidase, and a "Polypeptidase." In this system there are no carboxypolypeptidases nor are there enzymes hydrolyzing chains of glycine molecules.

In the case of *C. botulinum* certain experiments have been reported of potential significance. Stark, Sherman and Stark (1928a, b) allowed sterile filtrates of cultures to act on sterile skimmed milk, purified casein, and yeast cells. It was found that when one part of toxic filtrate was mixed and incubated with 49 parts of milk a seven-fold increase in the M.L.D. per cubic centimeter content of the toxic mixture resulted. These results were confirmed and extended by Meyer (1928).

The apparent significance of the extracellular enzymes, plus the fact that no direct approach to this problem has been reported, where the enzymes have been materially inhibited and the toxin formation determined, gave impetus to the collection and presentation of relevant data. An examination of the proteolytic enzymes present in the surrounding medium during all phases of growth has been attempted.

Experimental

The first step taken was to determine the time of appearance of toxin and the extracellular proteolytic enzymes.

Experiment 1. Tubes containing 10 cc. of glucose-beef heart medium prepared as described in the introduction were inoculated with a suspension of dried spores. The inoculum was small enough so that no change in turbidity of the medium resulted. At stated periods tubes were removed from the series, centrifuged

to remove the organisms, and the latter removed and washed in saline. Before the final washing and centrifugalization, the organisms were transferred to conical micro-Kjeldahl centrifuge flasks. The sediment was then resuspended in 1 cc. distilled water.

The Kjeldahl method for determining nitrogen, modified by Pregl (1930), was used to determine the bacterial nitrogen. One cubic centimeter of concentrated sulfuric acid was added to the suspension, which also received a knife-point of a mixture of copper and potassium sulfate (1:2). It was also found advisable to accept Mueller's recommendation (1935) that 4 drops of 30 per cent hydrogen peroxide be added three times during the digestion. The distillations were carried out in an all-glass apparatus designed and made by Mr. Cummings of the University Chemistry Department.

On the supernatant fluid from the centrifuged cultures determinations of proteinase, aminopolypeptidase, dipeptidase, and "polypeptidase" were made.

Another aliquot of the same supernatant fluid was diluted in a series of 10-fold dilutions and the toxin concentration determined in terms of M.L.D. per cubic centimeter by mouse inoculation. Duplicate mice were used for each dilution. Five-tenths cubic centimeter of each dilution was inoculated intraperitoneally and the amount required to kill a 20 gram mouse in 48 hours determined as closely as possible.

The composite data for a typical run are presented in table 8.

It will be observed that maximum bacterial nitrogen is attained around the 27th hour, after which a slow decline occurs, accompanied by a visible decrease in turbidity of the culture fluid. The nitrogen values level off and remain fairly constant thereafter.

Toxin begins to appear as early as the 21st hour of growth, reaching a peak long after the bacterial nitrogen maximum, and, it will be noticed, during the phase of visible autolysis.

The proteinase parallels the bacterial nitrogen curve, and appears about 6 hours before toxin is detectable. On the other hand the lower peptidases do not appear in appreciable amount until long after the toxin has appeared. One would be led to

believe that, if any of the enzymes might bear a causal relationship the proteinase would bear further investigation.

Experiment 2. Sodium cyanide solutions were prepared in M/15 phosphate buffer at pH 7.0. Ten-cubic-centimeter tubes of glucose-beef heart medium were adjusted to contain varying

TABLE 8

GROWTH	BACTERIAL NITROGEN PER 100 CC.	TOXIN (M.L.D. PER CC.)	PROTEINASE (VISCOSITY CHANGE)	AMINOPOLYPEPTIDASE	DIPEPTIDASE	POLYPEPTIDASE (CC. N/5 KOH)
hours	*mgm.*		*per cent*	*(per cent hydrolysis)*	*(per cent hydrolysis)*	
7	0.33	0	0	0	0	0
	0.50					
15	1.35	0	1.3	0	0	0
	1.68					
18	2.94	0	6.9	0	0	0
	3.20					
21	6.65	20	23.4	1	0	0.1
	6.65					
24	8.40	80	38.6	1	6	0.1
	8.39					
27	10.8	160	55.1	3	5	0.4
30	9.38	400	49.4	3	3	0.4
	9.38					
33	8.82	480	60.5	3	3	0.4
	8.86					
36	7.56	800	60.5	5	6	0.5
	7.59					
45	6.65	2,000	57		18	0.75
	6.58					
60	6.33	8,000	54.1	8	30	0.56
72	6.30	12,000	60	14		0.48
	6.37					
84	6.16	6,000	50.5	22	43	0.32
	6.11					
96	6.37	12,000	58.8	38	60	0.35
144				60	73	0.25

amounts of the cyanide salt and were then inoculated with a heated spore suspension. Sets of ten tubes per concentration of cyanide were used.

In 30 hours, tubes containing 0.01 mol, and less, cyanide per cubic centimeter medium showed growth equal to that in the

control tubes containing no cyanide. Tubes containing more than 0.01 mol sodium cyanide per cubic centimeter medium eventually showed growth but the appearance of this growth was sometimes delayed for 8–10 days. 0.02 mol was the upper limit of cyanide concentration (per cubic centimeter medium), above which no growth ever occurred.

Only tubes showing growth as soon as the control series (with no cyanide) were tested inasmuch as it was desired to simulate natural conditions as much as possible.

The proteinase tests were carried out in the usual manner with gelatin solution, at pH 7.0.

TABLE 9

Effect of sodium cyanide on production of proteinase

PROTEINASE PREPARATION	PER CENT CHANGE IN VISCOSITY
1. Untreated culture	83.5
2. Secreted in presence of 0.005 Mol per cc	77.5
3. Secreted in presence of .008 Mol per cc	52.3
4. Secreted in presence of 0.01 Mol per cc	28.3
5. Heat-inactivated	0.0
"Inactivation" by sodium cyanide in medium:	
0.005 Mol	7.2 per cent
0.008 Mol	38.3 per cent
0.01 Mol	66.2 per cent

The toxin determinations were made for M.L.D. per cubic centimeter of medium. Inasmuch as the tubes with 0.005, 0.008 and 0.01 mol sodium cyanide allowed growth at a rate equal to that of the control tubes, they were chosen for assay. The proteinase tests are presented in table 9.

That the proteinase was not simply altered in its rate of appearance rather than in the total amount produced was tested by examining over a period of 96 hours the proteinase content of the medium. The maximal point was reached in every case around the 30th hour with no further increase.

If the extracellular proteinase is directly concerned with toxin production, then tubes containing 0.008 and 0.01 mol. sodium

cyanide per cubic centimeter of medium should show a decreased amount of toxin, around the 96th hour when it is usually maximum in this medium. Such did not prove to be the case, however, as the data in table 10 indicate.

Tubes in which the proteinase showed 60 per cent inhibition or reduction in concentration were inoculated in 10-fold dilutions into mice in the usual manner. Control mice received antitoxin to check the possible effect of the cyanide and heat-inactivated toxin to further check the natures of the killing agent. A third series of mice received the supernatant fluid from cultures grown in the absence of cyanide.

The complete similarly in picture between the cultures grown in the presence and absence of sodium cyanide was confirmed

TABLE 10

TOXIN DILUTION (0.5 cc.)	CYANIDE-TREATED CULTURES	UNTREATED CULTURES	"HEAT-KILLED" TOXIN	ANTITOXIN-PROTECTED
10^{-2}	Dead—18 hours	Dead—18 hours	No symptoms	No symptoms
10^{-3}	Dead—26 hours	Dead—28 hours	No symptoms	No symptoms
10^{-4}	Dead—45 hours	Dead—43 hours	No symptoms	No symptoms
10^{-5}	Dead—55 hours	Dead—50 hours	No symptoms	No symptoms
10^{-6}	Symptoms severe	Symptoms mild	No symptoms	No symptoms
10^{-7}	No symptoms	No symptoms	No symptoms	No symptoms

on several repetitions. It was therefore concluded that a 60 per cent reduction in the proteinase activity of the extracellular system does not cause a reduction in the toxin concentration detectable in this manner. It remains to test this assumption again on very much larger numbers of mice for statistical evaluation.

Experiment 3. The fact that the efficiency of the bacterial enzyme systems may exert a masking influence, and that the 40 per cent remaining activity of the enzyme may still account for toxin production was tested by examining the distribution of the proteinase in types of *C. botulinum.*

Four "B" types, two "A" types, a "C" and a "D" types were examined. The data are presented in table 11.

For comparison, strains 526 C, 237 B, D, and 63 B were selected

for toxin determinations. Titrations of the supernatant fluid from 96-hour-old cultures in mice again failed to show any correlation with the proteinase activity as the data in table 12 indicate.

The cultures from Type D and strain 237 B show on one hand maximal deviation in enzyme activity and on the other a quantitative similarity in toxin-production ability. Strain 63 B

TABLE 11

Proteinase distribution

STRAIN	PER CENT CHANGE IN VISCOSITY IN 30 MINUTES BY 1 CC. CULTURE FLUID	
	24 hour culture	48 hour culture
237 (B)	83.6	81.0
34 (B)	74.5	39.3
London (A)	63.9	73.4
63 (B)	59.0	58.3
69 (A)	54.0	
6 (B)	30.3	43.9
526 (C)	9.8	0.0
(D)	0	0.0

TABLE 12

Distribution data of proteinase and toxin

STRAIN	PROTEINASE ACTIVITY	M.L.D. PER CC.
237	83.6	200,000
526	9.8	20,000
D	0	200,000
63	59	20,000

showing a moderately strong proteinase belongs in the same class toxigenically speaking with 526 C, which is practically inert as far as protein-splitting ability is concerned.

It would appear therefore very probable that the residual activity of the extracellular proteinase in cyanide-treated cultures was not a factor in toxin production but more probably concerned the maintenance of the bacterial "*status quo*."

The question concerning the designation of a toxin as an exo-

toxin or an endotoxin and the implications arising from such terms have recently been brought to attention once more.

The neurotoxins of *Corynebacterium diphtheriae*, *Clostridium tetani* and *Clostridium botulinum* have been generally accepted as classical examples of exotoxins. It is certainly true, in addition, that these toxins possess characteristics which at once distinguish them from the endotoxins of the cholera vibrio and the meningococcus. However, the true state of affairs in designating these toxins as endotoxins or exotoxins has been aptly summarized by Topley (1933) in the statement that "although this broad distinction can be made by comparing typical endotoxins with typical exotoxins, our difficulties become great if we attempt to assign each bacterial product to its correct group. . . ."

The data on the time of appearance of diphtheria, tetanus and botulinus toxins show that these toxins do not appear until the cells have reached the maximum growth phase, and, in fact, as our results show, botulinus toxin appears maximally during the phase when most of the cells are autolyzing.

The results of Eaton (1936a, b, 1937) have shown almost conclusively that the diphtheria toxin is a protein, which is heat-coagulable and which can be denatured by acids. Pappenheimer (1937a, b, c) has studied the purification of diphtheria toxin in a far simpler medium and also believes that the diphtheria toxin is a protein. Both investigators have isolated this toxic protein from media in which no protein tests were given before inoculation. The period of incubation before the toxin was harvested was around five to six days, enough time to elapse for considerable lysis of cells if reference is made to a paper by Moloney and Hanna (1921) and to the more recent and accurate studies of Mueller (1935).

The evidence indicates that diphtheria toxin is formed inside of the cells and is set free on the death and destruction of the cells.

Our results with botulinus toxin also point to its intracellular formation on the basis of the rise in M.L.D. when visible autolysis occurs. Furthermore, the difficulty of obtaining atoxic sus-

pensions of diphtheria and botulinus bacilli by washing is well-known. With diphtheria it is possible to produce anti-bacterial sera which are not antitoxic, as Eaton (1936b) succeeded in demonstrating. However, in the case of botulinus, Thom, Edmondson and Giltner (1919), Burke, Elder and Pischel (1921) and Nelson (1927) have stated that it is impossible to prepare botulinus bacilli or spores suspensions free of toxin, even after fifteen to twenty washings. In fact, Nelson (1927) found that a mass of botulinus bacilli could be extremely toxic despite the atoxicity of the broth fluid in which they had grown.

It would appear therefore that, with *C. botulinum*, one is dealing with an endotoxin, or at least with a toxin the locus of production of which is inside the cell. This change in conception does not in fact complicate the toxin make-up of bacteria because Boivin, Mesrobeaunu, Topley *et al* have shown that the classical endotoxins are very closely related, if not identical with, the somatic antigen. The toxin production of bacteria can therefore be pictured as occurring entirely within the cell. The true non-antigenic endotoxins may be considered intimately bound or identical with the soma whereas the characteristic antitoxinogenic toxins, also produced intracellularly, may be considered as substances to which the cell membrane is impermeable during the life of the cell, and which are distinct from the somatic antigens of the cell.

SUMMARY

1. The extracellular proteolytic system in relation to toxin production was studied. It was found that a "proteinase," acting on gelatin and casein was secreted into the medium. This enzyme acted optimally at pH 7.0. It was inactivated by sodium cyanide, cysteine, hydrogen peroxide and copper salts.

2. The lower members in the proteolytic system included a polypeptidase acting on Witte's peptone, an aminopolypeptidase, hydrolyzing leucyl-glycyl glycine and a dipeptidase, hydrolyzing leucyl glycine. The optimal pH values for these enzymes was 7.8–8.0.

3. Experiments on rate of appearance revealed that the

proteinase appeared first in the culture fluid, paralleling the trend of bacterial nitrogen. It also appeared at least six hours before the toxin could be demonstrated.

4. The polypeptidases and dipeptidase appeared simultaneously with toxin but, whereas the toxin reached its maximum around the ninety-sixth hour, these enzymes slowly increased during the first six days.

5. When, during the normal growth of the organisms, the proteinase was inactivated by the presence of sodium cyanide in the medium, the production of toxin was unchanged and unaltered.

6. Various types of *Clostridium botulinum* showed no correlation between the extracellular proteolytic enzymes and toxin production. Types "C" and "D" produced large amounts of toxin, comparable with the proteolytic type "A" in the absence of any detectable quantities of proteinase. Strains of type "B" on the other hand produced amounts of proteinase comparable to that of type "A" but produced approximately one-tenth as much toxin.

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