ACTIVATION OF *CLOSTRIDIUM BOTULINUM* TYPE E TOXIN BY TRYPSIN

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Since 1936, when the type E strain of *Clostridium botulinum* was first established as a result of the work of Gunnison *et al.* (1936), 17 outbreaks of Type E botulism have been reported, involving 69 persons, of whom 21 died (Dolman and Chang, 1953; Dolman *et al.*, 1955). Although the large proportion of fatalities suggests that this organism is highly toxigenic, published reports indicate that in the laboratory type E strains produce very low toxicities for mice, as compared with types A, B, C and D. The highest titer of type E toxin obtained by conventional methods was reported by Dolman, who obtained 4000 MLD/ml by growth of the VH strain in a peptone-beef infusion broth (Dolman *et al.*, 1950). When this strain was grown by the cellophane bag method, titers that ranged from 10,000 to 50,000 mouse MLD's/ml were obtained (Barron and Reed, 1954).

Investigations in this laboratory on the type E strain were directed toward development of a toxoid satisfactory for immunization. An inexpensive medium was required in which toxin production was consistent, and studies were carried out to determine the effect of various peptones, hydrolyzed caseins, amino acids, yeast extracts, and carbohydrates upon toxin production in bottle-grown cultures. Titers consistently greater than 5000 mouse intraperitoneal LD_{50}/ml were not obtained. Occasionally, a type E culture contaminated with unidentified organisms of the genus *Bacillus* produced higher titers of type E toxin than the pure culture. Devlin and Soderstrom (1954, *personal communication*), of the Parke, Davis and Company Research Laboratories, made similar observations and noted that when the toxin in these cultures was fractionated, yields greatly in excess of 100 per cent were frequently obtained.

These observations suggested the presence in the cultures of an inactive toxin or protoxin similar to that described by Turner and Rodwell (1943*a*, *b*) for the epsilon toxin of *Clostridium perfringens* (*C. welchii*) type D, and by Ross *et al.* (1949) for the iota toxin of *Clostridium perfringens* type E. These investigators reported that the toxicity of crude preparations of these toxins was greatly increased by the action of trypsin. We have found that the type E toxin of *C. botulinum* may be activated in a similar manner, and the present paper describes certain aspects of the activation phenomenon.

MATERIALS AND METHODS

Strains. Five strains of *C. botulinum* type E were investigated. Strain VH, sent to us by Dr. C. E. Dolman, had been isolated from an outbreak of human botulism in Canada (Dolman, *et al.*, 1950). Strain P-36, sent to us by Dr. A. R. Prévot, had been isolated from a freshwater perch in France (Prévot and Huet, 1951). Strain 8 (New York State 37416) and strain 30-b (New York State 37417) were isolated in Russia and identified by Gunnison *et al.* (1936). Strain Salmon had been isolated from Labrador canned salmon (Hazen, 1938). The latter three strains were sent to us by Dr. E. L. Hazen.

The stock strains were grown for 48 hr at 30 C in 200-ml volumes of a medium composed of beef infusion, 1.0 per cent peptone, and chopped meat. After growth, the culture supernatants were distributed into small tubes, quick frozen at −60 C, and stored in a deepfreeze at −20 C. Storage of the stock culture for over 1 year under those conditions has had no adverse effect on toxigenicity and viability.

Medium. The medium for toxin production was composed of 2.0 per cent proteose peptone (Difco), 2.0 per cent yeast extract (Baltimore Biological Lab.) and 1.0 per cent dextrin (Difco). The medium was adjusted to pH 7.2 before autoclaving. The dextrin was autoclaved separately as a 20 per cent solution and added aseptically.

Production of toxin. For production of toxin, 2 to 3 ml of the stock culture was inoculated into 15 ml of the chopped meat infusion-peptone medium described above and incubated at 30 C for 24 hr. Approximately 10 ml of this culture was transferred to a 150-ml volume of the production medium which was grown for 24 hr at

30 C. This culture was then used to inoculate a 3-L volume of the production medium contained in a 4-L bottle. The final cultures were grown at 30 C for 5 days.

Toxicity titrations. Toxin was diluted in a buffer composed of gelatin, 0.2 per cent; dibasic sodium phosphate, 0.4 per cent; and hydrochloric acid to pH 6.2. White mice weighing 18 to 20 g were injected intraperitoneally with 0.5-ml aliquots of the dilutions of toxin and observed for 4 days. The number of mice per dilution ranged from 4 to 8. For estimation of oral toxicity, mice were force-fed with 1.0-ml amounts of the dilution. Guinea pigs were injected subcutaneously with 1.0-ml aliquots of the dilutions. The LD_{50} was calculated by a graphic probit method (Weiss, 1948).

Activation with trypsin. Prior to the addition of enzyme, 20 ml of toxin were adjusted to the specified pH at 25 C, the temperature was raised to the desired value and trypsin was added. Incubation was carried out in a water bath. Crude trypsin was obtained from Difco Laboratories, and crystalline trypsin and soy bean trypsin inhibitor from Armour Laboratories.

EXPERIMENTAL RESULTS

The effect of trypsin on the toxicities of cultures of a representative group of *C. botulinum* type E strains is shown in table 1. These strains were grown in the production medium for 5 days at 30 C and were not clarified before assay. The primary LD_{50}/ml represents the toxicity assay on cultures after they were adjusted to pH 6.0 and incubated at 37 C for 45 min. These strains varied slightly in their production of toxin, but all produced toxin of a low potency when assayed in mice. Incubation at 37 C for 45 min did not affect the culture toxin. The activated LD_{50}/ml represents the toxicity assay on cultures that were adjusted to pH 6.0 and incubated at 37 C for 45 min in the presence of 1.0 per cent trypsin (Difco 1:250). Under these conditions, a marked increase in toxicity was noted with cultures of all five strains. The ratio of the activated LD_{50}/ml to the primary LD_{50}/ml is referred to as the activation ratio. This ratio ranged from 12 to 47, and the maximum toxicity obtained was 180,000 LD_{50}/ml. Since optimum conditions for growth of the cultures and activation of the toxin may not have been used, this may not represent the highest culture toxicity possible for the type E strains. Control solutions containing gelatin phosphate buffer and 1.0 per cent trypsin were not toxic at a 1:2 dilution. For subsequent experimental studies, the Dolman VH strain was used.

TABLE 1

Effect of trypsin on toxicity of cultures of various Clostridium botulinum type E strains

Strain	Mouse Intraperitoneal LD_{50}/ml		Activation Ratio
	Primary	Activated	
VH	5800	180,000	31
8	3000	140,000	47
30-b	7700	120,000	15
P-36	4600	130,000	28
Salmon	3800	46,000	12

TABLE 2

Effect of incubation time on toxin production

Time	Culture	Mouse Intraperitoneal LD_{50}/ml	
		Primary	Activated
days	*pH*		
1	6.7	400	12,000
2	6.4	1500	52,000
3	5.9	3000	64,000
4	5.7	3700	120,000
5	5.4	4900	180,000
6	5.3	5300	260,000
7	5.2	4600	230,000
34	5.2	2600	130,000

Treatment with trypsin also increased the toxicity of cultures that had been clarified by centrifugation. The activity of the trypsin-treated clarified culture was approximately the same as that obtained with the trypsin-treated whole culture, showing that liberation of toxin from the bacterial cells was not the basis of the increase in toxicity.

The effect of incubation time on production of toxin was studied, and these results are shown in table 2. The conditions used for activating the toxin were the following: 1.0 per cent trypsin (Difco 1:250), pH 6.0, and incubation at 37 C for 45 min. The data from several experiments showed that the cultures reached their maximum toxicity as measured by the primary and activated LD_{50} after incubation for 5 to 7 days. The pH of the culture reached essentially its final value after the same period. In a single experi-

TABLE 3

Effect of trypsin concentration and pH on activation of toxin at 37 C for 45 min

pH	Mouse Intraperitoneal LD_{50}/ml			
	Per cent trypsin (1:250)			
	0	0.05	0.1	1.0
5.0		16,000	<40,000	96,000
5.5	5,200	32,000	<40,000	220,000
6.0		52,000	120,000	220,000
6.5	5,800	110,000	110,000	120,000
7.0		24,000	<8,000	10,000
7.5	5,800	5,600	3,000	4,000

TABLE 4

Effect of pH and incubation time at 37 C on activation of toxin by 0.1 per cent trypsin

Time	Mouse Intraperitoneal LD_{50}/ml				
	pH				
	5.0	5.5	6.0	6.5	7.0
min					
<1*	5,800	12,000	12,000	2,900	8,000
5	10,000	20,000	29,000	20,000	29,000
15	10,000	40,000	58,000	20,000	58,000
30	29,000	80,000	120,000	80,000	20,000
60	29,000	120,000	200,000	120,000	20,000
120	120,000	280,000	230,000	230,000	15,000
Controls (without trypsin)					
30	2,900	2,900	2,600	3,200	2,900
120	2,900	3,200	3,200	2,900	2,600

* Sample removed as soon as possible after addition of trypsin.

ment, after incubation for 34 days the primary and activated toxicities had decreased to approximately one-half the maximum, indicating that both forms of the toxin are relatively stable in the culture.

The effect of trypsin concentration and pH on activation of nonclarified whole culture incubated 45 min at 37 C is shown in table 3. The greatest increase in toxicity occurred in the pH range 5.5 to 6.0 with 1.0 per cent trypsin. With 0.1 per cent trypsin, the optimum was near pH 6.0, and with 0.05 per cent trypsin the optimum was pH 6.5. The increase in toxicity was considerably less in the high and low pH range. Data not shown here indicated that 2.0 per cent trypsin gave the same toxicity as 1.0 per cent trypsin at pH 6.0.

The effect of pH and incubation period at 37 C in the presence of 0.1 per cent trypsin on activation of clarified culture toxin is shown in table 4. The optimum pH appeared to be between 5.5 and 6.5, and in this pH range the time for the maximum activation was approximately 60 to 120 min. At pH 7.0, complete activation (in terms of that obtained at pH 5.5 to 6.5) did not occur. The greatest activation at pH 7.0 occurred at approximately 15 min, and the toxicity decreased somewhat on further incubation. The toxin was stable at this pH in the absence of trypsin, so it would appear that at this pH activation and destruction of toxin take place simultaneously.

The course of the activation reaction was studied in greater detail at pH 5.2 and 6.0; at 30, 37, and 40 C; and with 0.1, 0.3, and 1.0 per cent trypsin. The conditions were selected to include the temperature of incubation and the pH of the culture after growth. One preparation of whole culture containing 5800 LD_{50}/ml before activation was used in all the experiments except those at pH 6.0 and 37 C. The results are presented in table 5.

The most rapid and complete activation occurred at pH 6.0. At pH 5.2 the reaction proceeded more slowly, but with the higher trypsin concentrations the toxicity reached the same level as at pH 6.0. In general the reaction proceeded more rapidly at the higher temperatures. The significance of the decrease in rate between 37 and 40 C at pH 6.0 may be questionable because of the use of different toxin preparations. On the basis of these observations the conditions selected for routine activation of whole culture were the following: 0.1 per cent trypsin, pH 6.0, and incubation at 37 C for 75 min.

The activation of toxin by crystalline trypsin was studied. A 0.02 per cent solution of crystalline trypsin was prepared in water and adjusted to pH 6.0 with dilute sodium hydroxide solution. Equal parts of clarified culture toxin at pH 6.0 and trypsin solution were mixed and incubated at 37 C. The activation obtained with crystalline trypsin was similar to that obtained with the crude product. The time required for maximum activation was approximately 60 min.

The effect of crystalline soy bean inhibitor on activation of the toxin by crystalline trypsin was

TABLE 5

Effect of temperature, pH, trypsin concentration and incubation time on activation of toxin

Time	Trypsin	Mouse Intraperitoneal LD_{50}/ml (in thousands)					
		pH 5.2			pH 6.0		
		30 C	37 C	40 C	30 C	37 C*	40 C
min	%						
15	0.1			12	20	58	29
30		8	13	15	29	120	64
60		16	29	20	58	200	120
120		32	58	58	120	230	130
240		52	120	130	120		200
1200		80	130	130	160		120
15	0.3			26	29	120	58
30		13	23	52	58	230	120
60		29	58	58	130	230	230
120		58	120	120	120	230	200
240		52	130	120	120		230
1200		160	230	130	120		130
15	1.0			64	120	230	160
30		40	80	80	120	230	200
60		120	160	230	120	230	130
120		130	230	200	230	230	200
240		230	160	230	230		160
1200		200	230	200	200		100

* Data were obtained in a separate experiment with a similar preparation of toxin.

investigated. A 0.02 per cent solution of crystalline soy bean inhibitor was prepared in 0.0025 M hydrochloric acid solution and adjusted to pH 6.0 with weak sodium hydroxide solution (Kunitz, 1948). Equal portions of inhibitor solution and crystalline trypsin solution were mixed and added to the clarified culture toxin at pH 6.0. The mixture was incubated at 37 C and assayed for toxicity after 45 min. When inhibitor was added to the trypsin before the addition of toxin, no activation took place.

The effect of trypsin on the toxicity of the type E culture for mice and guinea pigs by different routes is shown in table 6. There was an approximately 50-fold increase in toxicity for mice by the intraperitoneal route when culture toxin was activated with trypsin, but by the oral route there was no significant increase in toxicity. The LD_{50}/ml of nonactivated toxin was approximately 200 times greater by the intraperitoneal route than by the oral route. The LD_{50}/ml of activated toxin was approximately 10,000 times greater by the intraperitoneal route than by the oral route. The culture toxin was activated by trypsin as measured by subcutaneous injection into guinea pigs, and the activation ratio was essentially the same as the activation ratio for mice by the intraperitoneal route.

Type E toxin purified according to a procedure to be described in a subsequent paper was increased in activity after the addition of trypsin. The purification procedure involves precipitation of toxin from whole culture by ethanol in the cold, extraction of toxin from the ethanol precipitate with calcium chloride solution, and reprecipitation twice with ethanol. The purified fraction contained 64,000 mouse LD_{50}/ml. When this toxin fraction was treated with 0.1 per cent trypsin (Difco 1:250) at pH 6.0 for 45 min, the resulting activity was 3.7×10^6 LD_{50}/ml. When the whole culture was activated with trypsin and then purified by the same procedures, the final purified fraction contained 1.6×10^6 LD_{50}/ml. These data indicate that purified toxin may be

TABLE 6

Effect of trypsin treatment on toxicity of type E culture for guinea pigs and mice by different routes

	Toxin I	Toxin II
Mouse oral LD_{50}/ml before activation	20	10
Mouse oral LD_{50}/ml after activation	20	10
Ratio	1	1
Mouse IP LD_{50}/ml before activation	3,600	2,400
Mouse IP LD_{50}/ml after activation	130,000	160,000
Ratio	36	67
Mouse IP/oral ratio before activation	180	240
Mouse IP/oral ratio after activation	6,500	16,000
Guinea pig SC LD_{50}/ml before activation	75	80
Guinea pig SC LD_{50}/ml after activation	3,000	2,900
Ratio	40	36

activated in the same manner as crude toxin, and that activated crude toxin may be purified in the same manner as untreated crude toxin. Further study of the fractionation of activated toxin is in progress.

The untreated and trypsin-treated toxin both before and after purification were neutralized by type E antitoxin[1] as demonstrated by *in vivo* neutralization titration in mice. Thus the toxicity that resulted from treatment with trypsin had the same specificity as the primary toxicity.

DISCUSSION

The present observations provide an explanation for the discrepancy between the low toxicity of type E cultures in the laboratory and their ability to produce the highly fatal toxemia associated with human type E botulism. Trypsin-treated cultures injected intraperitoneally into mice showed a striking increase in toxic activity as compared with untreated cultures. When these same cultures were administered orally to mice there was no significant difference in toxic activity. This suggests that type E toxin ingested orally is activated in the alimentary tract before adsorption. Evidently the conditions that determine the degree of activation of the toxin are of major importance in determining intraperitoneal toxicity, but have little effect on oral toxicity. Accordingly, measurement of the intraperitoneal toxicity of incompletely activated cultures will provide misleading information regarding the relative oral toxigenicity of strains, and relative toxin production under different cultural conditions. Cultures of the organism in media and in foods may vary widely in the proportion of the potential toxicity that may be measured by intraperitoneal assay in mice, because the composition of the growth medium may influence the activation reaction and determine the proportion of total toxin that is present in the active form.

The data provide no indication of the mechanism of the activation process. The time required for the increase in toxicity, and the inhibition of the reaction by trypsin inhibitor, indicate that the activation is enzymatic. Under the conditions studied, the optimum pH is considerably more acid than the optimum for the usual proteolytic activity of trypsin, evidently because the rate of destruction of the toxin by trypsin increases rapidly above pH 6.5.

It is probable that the toxin is elaborated by the cell as an inactive or relatively inactive precursor or protoxin, and that trypsin converts the protoxin to active toxin. This concept is analogous with the elaboration of enzymes as proenzymes, and has been suggested to explain proteolytic activation of the epsilon toxin of *C. perfringens* type D (Turner and Rodwell, 1943*a*, *b*) and of the iota toxin of *C. perfringens* type E (Ross *et al.*, 1949). It appears improbable that trypsin acts on some other constituent to yield a potentiating factor, because the toxin is readily activated after considerable purification, and may be purified after trypsin treatment with only minor loss of toxicity.

Type E strains are regarded as only slightly proteolytic, and presumably this is the reason that only partial activation of the toxin occurs during growth. It is possible that with the more proteolytic types of *C. botulinum*, activation occurs during growth of the culture. Should activation prove to be a general phenomenon in the various types, established concepts and procedures will require reexamination.

SUMMARY

The intraperitoneal toxicity for mice of cultures of five strains of *Clostridium botulinum* type E was increased 12- to 47-fold by incubation with trypsin. The toxicity for guinea pigs by subcutaneous injection was increased similarly. Treatment with trypsin did not alter the toxicity for mice by the oral route. Conditions for activation were investigated; the optimum pH at 37 C was approximately 6.0. The reaction was inhibited completely by soy bean trypsin inhibitor. Clarified culture and partially purified toxin were activated in a similar manner. Activated and nonactivated toxins were fractionated by the same procedure, and both purified toxins were neutralized by type E antitoxin. Certain implications of the results were discussed.

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