STUDIES ON TOXIN PRODUCTION OF *CLOSTRIDIUM BOTULINUM* TYPE E

III. Characterization of Toxin Precursor

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The toxicity resulting from the treatment of young cells of *Clostridium botulinum* type E with a proteolytic enzyme produced by a contaminant (strain 13) suggests the change of an inactive toxin precursor within the cells to an active toxin (Sakaguchi and Tohyama, 1955*a*, *b*). A rapid activation of type E toxin results with trypsin treatment (Duff *et al.*, 1956). The "unusually potent toxin" produced in a mixed culture of a toxigenic and a nontoxigenic but proteolytic mutant (Dolman, 1953) has been ascribed to a similar phenomenon (Dolman, 1957).

Thus the possibilities of increase in toxicity by activation in the digestive tract or by proteolytic enzymes in uncooked fish, make the activation phenomenon of type E botulinum toxin of practical and academic interest.

The mechanism of the activation phenomenon is unknown. If the increase of toxin potency by the proteolytic enzymes is due to the enzymatic cleavage of certain linkage(s) of a toxin precursor, which is not itself toxin, then a nontoxic material capable of being transformed into a toxic substance should be demonstrable. The isolation of this precursor moiety from young cells of *C. botulinum* type E and some of its properties are presented in this paper. The term "precursor" is used to designate the substrate which, when acted upon by trypsin, results in the formation of the toxic product. The term "activation" is used to mean the increase in toxicity after treatment with proteolytic enzymes.

MATERIALS AND METHODS

Strains. Three strains of *C. botulinum* type E were used, strain Tenno was isolated by Sakaguchi *et al.* (1954) from gilthead izushi. Strain VH was isolated by Dolman *et al.* (1950) from an outbreak in Canada. Strain Iwanai was isolated in Japan in 1951 from herring izushi by Nakamura *et al.* (1956).

Culture media. The media used for obtaining bacterial cells were 0.5 per cent glucose, beef liver infusion broth, brain heart infusion broth (Difco), and V. F. broth (peptic digests of beef and liver). Seed cultures were obtained by growing organisms in liver-liver infusion broth for 1 day at 30 or 37 C. The inoculum ranged from 2 to 10 per cent of the test medium inoculated.

Activation. Trypsin (Merck) at pH 6.0 was used in a concentration of 1 per cent. Salt-free crystalline trypsin (Armour) was used at the same pH at a concentration of 0.01 per cent (Duff *et al.*, 1956). Incubation was at 37 C for 60 or 120 min.

Toxin assay. Toxin was diluted in a pH 6.0 solution made with one volume of 0.06 M phosphate buffer, three volumes of 0.85 per cent NaCl, and gelatin to make 0.02 per cent final concentration. Mice weighing approximately 15 g were injected intraperitoneally with 0.5 ml of toxin. The animals were observed for 4 days. Minimal lethal doses (MLD) were calculated from the highest dilution causing death of more than one half of the group.

Characterization of samples. Total phosphorus was determined by the method of Allen (1953). Deoxyribonucleic acid was determined by Dische's test modified by Burton (1956) with thymus deoxyribonucleic acid as a standard. Ribonucleic acid was calculated from a ribose value as determined by the orcinol-HCl test of Mejbaum (1953). Total carbohydrate was determined by the anthrone test and expressed in terms of glucose equivalent. The total protein content was determined by means of Folin phenol reagent according to Lowry *et al.* (1951) and expressed in terms of crystalline bovine serum albumin (Armour) equivalent. Protein nitrogen was determined by dividing the protein values by 6.25.

Starch electrophoresis. All separations were performed in 0.5 M acetate buffer at pH 6.0. A starch bed was prepared by pouring buffer-

washed starch slurry into a plastic starch bed holder (40 by 2 by 1.5 cm) fitted with filter paper at each end. One- or two-cm sections of starch were removed and dried. An appropriate amount of sample was dissolved in the buffer not exceeding 1.0 ml, mixed thoroughly with the dried starch, and replaced in the bed. Development was carried out in the refrigerator for 17 to 19 hr at a potential gradient of 8 to 10 v per cm. After separation, 2-cm sections were extracted with 5.0 ml of the acetate buffer and protein and toxin determined on each extract.

Activation of the sample before electrophoresis was made by dissolving the sample in 1.0 ml of buffer containing 100 μg or 1.0 mg of crystalline trypsin and incubating it for 60 min at 37 C. Activation of the starch extracts was accomplished by incubating an aliquot of each extract with an equal volume of 2 per cent crude trypsin solution in buffer.

EXPERIMENTAL RESULTS

Comparative activation of cells and supernatants. Although our previous results indicated that activation was mostly in the bacterial cells, the results of Duff *et al.* (1956) suggest otherwise. Therefore, activation of the cells and the culture supernatants were compared.

The three *C. botulinum* strains were grown at 37 and 30 C in glucose beef liver infusion for 7 days. Maximum growth occurred within 24 hr. Aliquots of the culture were removed daily and the cells removed by centrifugation at 1500 × G for 15 min. The cells were made to original volume with 0.06 M acetate buffer of pH 6.0 and the supernatants were adjusted to the same pH with NaOH. Both fractions were activated with crude trypsin for 2 hr at 37 C.

As can be seen from table 1, the toxin potency after activation is higher in the cellular fractions than in the corresponding supernatants except with VH grown at 37 C for longer than 2 days. The ratio of toxin titers after activation of cells to supernatant is largest with Tenno and smallest with VH which may indicate that the latter strain releases the precursor more readily into the medium than does Tenno.

Isolation of precursor. The Tenno strain was used in most cases for isolation of the toxin precursor because of the relatively large amount of precursor substance present in these cells. The isolation of the precursor is schematically shown in figure 1. The washed organisms (from step 2) were lyophilized or immediately subjected to extraction. From 21 to 59 per cent of the activity was sacrificed in the supernatant and wash waters,

TABLE 1

Comparative activation of cells and supernatant of cultures of Clostridium botulinum type E

Strain	Incubation Period	37 C			30 C		
		Supernatant		Cells after activation	Supernatant		Cells after activation
		Before activation	After activation		Before activation	After activation	
	days	*MLD/ml*			*MLD/ml*		
Tenno	1	—	20	200	—	200	2,000
	2	0	20	2,000	8	4,000	20,000
	3	—	80	2,000	—	4,000	20,000
	5	2	160	2,000	32	8,000	8,000
	7	—	20	800	—	4,000	8,000
Iwanai	1	—	80	800	—	800	8,000
	2	2	80	800	20	4,000	8,000
	3	—	160	1,600	—	8,000	—
	5	8	320	800	64	4,000	6,400
	7	4	80	320	320	200	6,400
VH	1	—	2,000	20,000	—	2,000	2,000
	2	320	16,000	2,000	80	8,000	20,000
	3	—	—	2,000	—	—	20,000
	5	200	8,000	4,000	160	16,000	20,000
	7	400	8,000	2,000	40	1,600	6,400

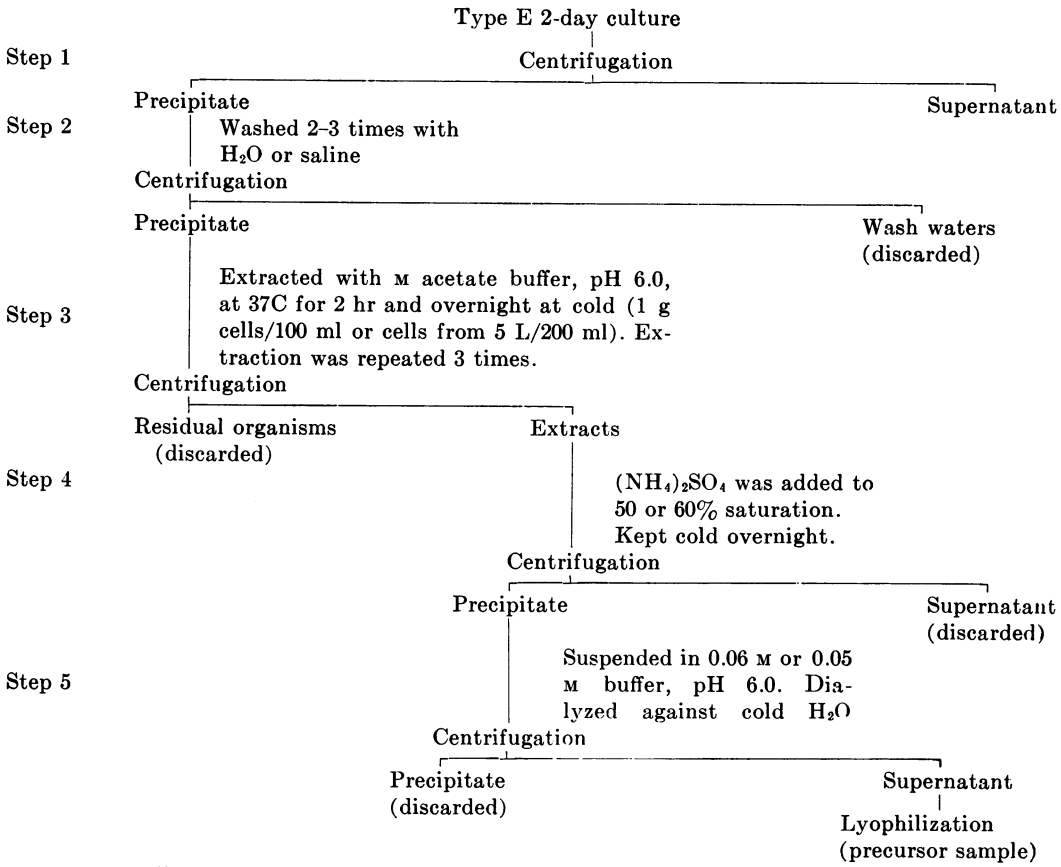

Figure 1. Procedures for obtaining precursor samples from bacterial cells.

TABLE 2

Characterization of precursor samples

Name of sample	Cell Origin			Supernatant Origin
Strain	7S26E-G "Tenno"	7S34F-1 "Tenno"	8S08-I "Tenno"	7S 48-A "VH"
Culture	37 C, in B-L-infusion	37 C, in B-L-infusion	30 C, in B-H-I-B (Difco)	30 C, in V.F. broth
Toxin potency:				
Before activation (MLD/mg N)	4.2×10^2	1.5×10^3	6.6×10^3	1.7×10^5
After activation (MLD/mg N)	1.2×10^5	1.5×10^5	5.2×10^6	2.1×10^6
Activation ratio	290	100	790	12
Protein (albumin equivalent) (%)	59.2	67.0	47.7	59.7
Total P (%)	3.0	2.5	3.2	0.6
Deoxyribonucleic acid (%)	0.1	0.2	0.2	0.3
Ribonucleic acid (%)	6.4	13.1	14.2	3.1
Carbohydrate (glucose equivalent) (%)	5.0	2.2	6.5	8.1
Yield	204 mg from 2 g cells (~ 2.5 L)	144 mg from 2 g cells (~ 2.6 L)	37 mg from 1 L culture	73 mg from 5.5 L culture

but 98 per cent of the substances reacting with the Folin phenol reagent were also eliminated, thus affording a 30-fold purification. From a batch of bacterial cells prepared from 5 L of culture, 15 per cent of the protein was obtained in the first extract, 3 per cent in the second, and 1 per cent in the third (step 3). The extraction of the activity was about 75 per cent. The activity from the cells was not increased when they were subjected to sonic oscillations or alternate freezing and thawing. Enzymatic digestion of the organisms by pepsin, papain, or chymotrypsin failed to increase toxin. Ammonium sulfate precipitation removed about 70 per cent of the activity and 35 per cent of the protein of the extract. Acid precipitation of precursor from M buffer extract was unsuccessful. The precipitate obtained during dialysis removed about 3 per cent activity and about 30 per cent of the protein.

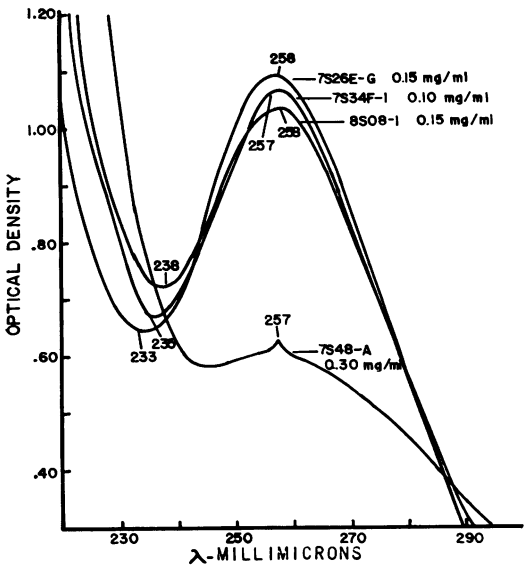

Figure 2. Ultraviolet absorption patterns of the precursor samples

The precursor present in the supernatant (table 2) was precipitated from 60 per cent saturated ammonium sulfate solution, dissolved in distilled water, and dialyzed. Any insoluble material was removed by centrifugation before lyophilization. The lyophilized precursor was dissolved in 0.05 M acetate buffer at pH 6.0 and precipitated from 40 per cent saturated ammonium sulfate solution. This precipitate contained 42 per cent of the activity of the first precipitate and 11 per cent of the protein.

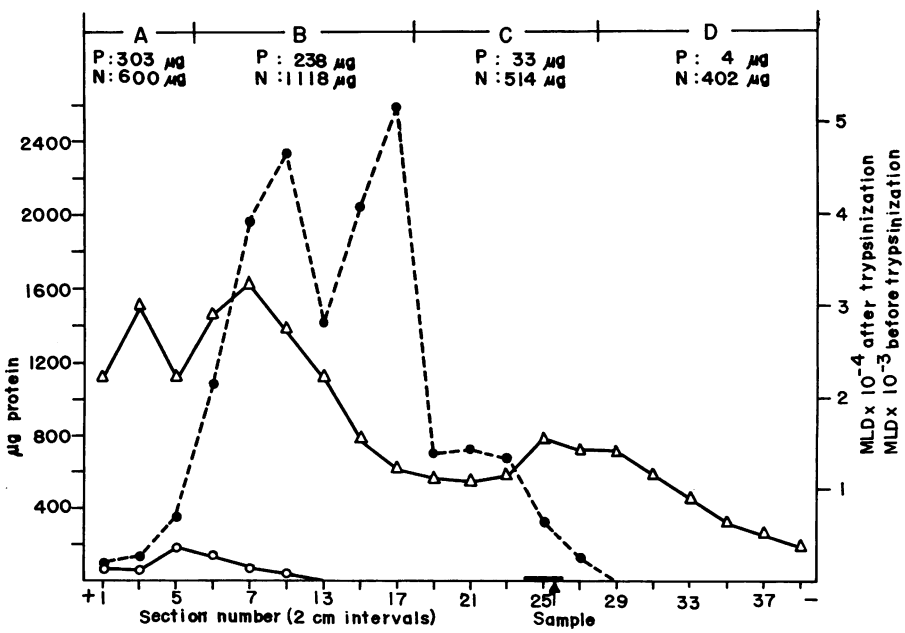

Figure 3. Starch electrophoresis of untreated 7S34F-1 (40 mg). Separation conditions: 350 v; 10 ma, 19 hr. △, µg protein; ○, MLD per mg protein without trypsinization; ●, MLD per mg protein after trypsinization.

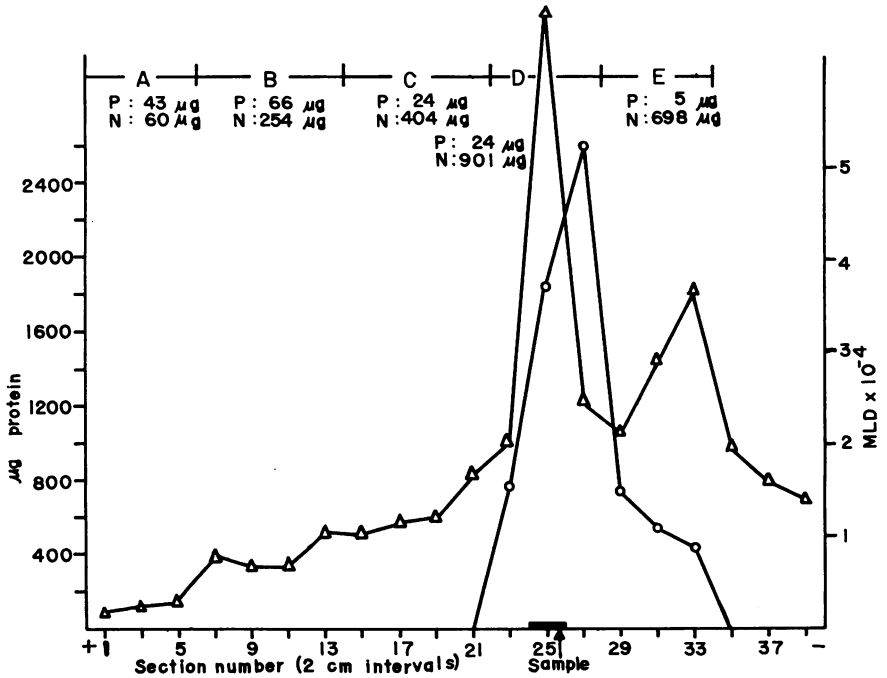

Figure 4. Starch electrophoresis of trypsinized 7S34F-1 (40 mg by 1 mg crystalline trypsin). Separation conditions: 350 v, 10 ma, 17 hr. △, µg protein; ○, MLD per mg protein.

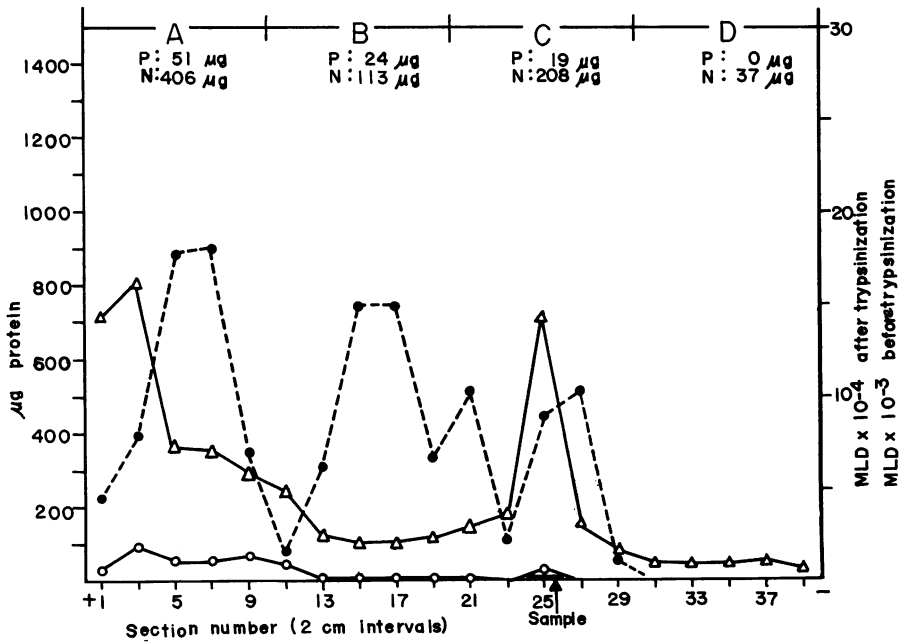

Figure 5. Starch electrophoresis of untreated 8S08-I (15 mg). Separation conditions: 325 v, 11 ma, 17 hr. △, µg protein; ○, MLD per mg protein without trypsinization; ●, MLD per mg protein after trypsinization.

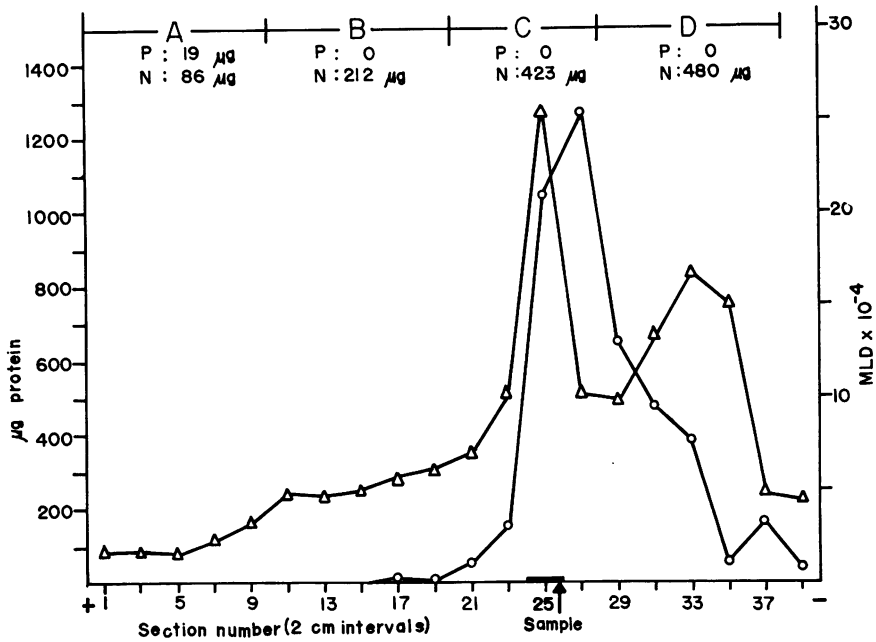

Figure 6. Starch electrophoresis of trypsinized 8S08-I (15 mg by 1 mg crystalline trypsin). Separation conditions: 325 v, 11 ma, 17 hr. △, μg protein; ○, MLD per mg protein.

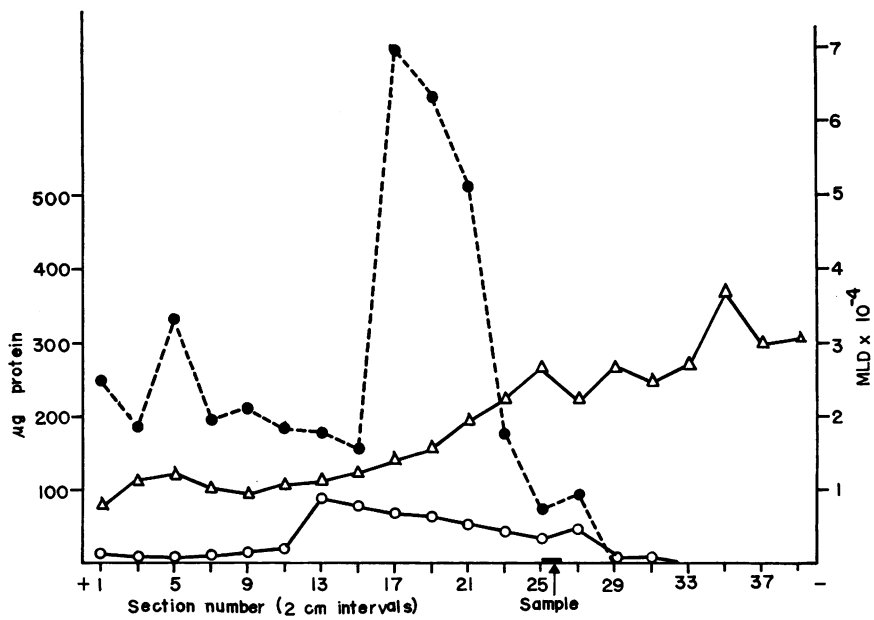

Figure 7. Starch electrophoresis of untreated 7S48-A (5 mg). Separation conditions: 350 v, 13 ma, 19 hr. △, μg protein, ○, MLD per mg protein without trypsinization; ●, MLD per mg protein after trypsinization.

Characterization of precursor samples. The characterization of the precursor samples is detailed in table 2. On the protein basis it can be estimated that the purification was enhanced 300- to 400-fold. The table and the ultraviolet absorption patterns for these samples (figure 2) indicate the presence of nucleic acids.

Starch electrophoresis of precursor samples.

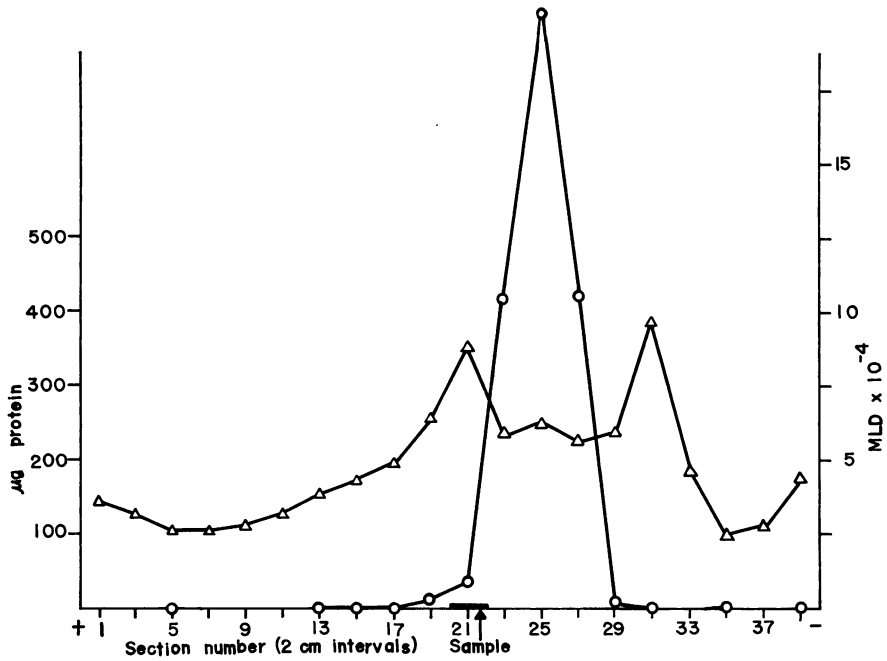

Figure 8. Starch electrophoresis of trypsinized 7S48-A (5 mg by 100 μg crystalline trypsin). Separation conditions: 350 v, 13 ma, 18 hr. △, μg protein; ○, MLD per mg protein.

Starch electrophoresis studies were made on the precursor samples as well as on the trypsin treated samples. The results are given in figures 3 to 8.

Less phosphorus was found associated with the toxin zones in the trypsin treated materials. The ultraviolet absorption at 257 to 258 μ indicated the absence of ribonucleic acid in these zones.

A slight purification was accomplished by starch electrophoresis with some of the samples; however, the recovery was usually very poor. Attempts to recover the precursor and toxin fractions by dialysis and lyophilization of the extracts resulted in almost complete loss of the activity.

DISCUSSION

Trypsin was found to be more effective in obtaining type E toxin from the bacterial cells than proteolytic enzymes from a contaminant (strain 13). Although Duff *et al.* (1956) found that liberation of toxin from bacterial cells was not the basis for the increase in toxicity, this and other work previously reported clearly demonstrates that trypsin or other activators do liberate toxin directly from the bacterial cells. This study indicates that there is a difference among strains with respect to this property and that Duff's conclusions may be based upon the strains which he used. There is an advantage in using young bacterial cells for liberating the toxin since by this method some components of the culture medium may be eliminated and by using young cells toxin may be harvested in a 2-day rather than a 5-day incubation period where the toxin is obtained from the supernatant fluid.

By centrifuging and washing the organisms two or three times, approximately 30-fold purification of the precursor can be accomplished. By precipitation of the protein 400-fold purification of the toxin was accomplished, which is approximately that obtained by Gordon *et al.* (1957). Attempts to disintegrate the bacterial cells did not aid in the extraction of the precursor. The precursor contained ribonucleic acid and probably represents a part of the cytoplasm located rather near the surface of the cells.

The electrophoretic experiments indicated that the precursor is acidic in nature and this property may be related to the presence of ribonucleic acid. Activation of type E toxin by trypsin may involve a change of charge from negative to neutral or positive which may be due to the splitting off of ribonucleic acid. Considerable

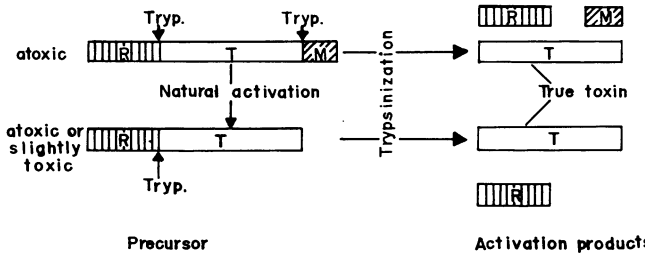

Figure 9. A proposed schema for possible mechanisms of activation of type E toxin by trypsin. *R*, a group which contains ribonucleic acid; *T*, toxin moiety; *M*, masking group.

toxicity was demonstrated in anionic fractions with the untreated precursor samples and no toxin was found in the catonic fractions. Assuming all toxin was of a neutral or basic nature, then 6×10^4 to 9×10^5 MLD of toxin should have been recovered in the cationic fractions in the electrophoretic experiments with untreated samples.

Possible interpretations of the experimental results may be: (1) Anionic toxin may represent activation in the body of the test animal. When foreign protein is injected intraperitoneally into mice there is an increase in neutrophilic leukocytes which contain proteolytic enzymes. This neutrophilic response occurs within a few hours and in sufficient time for the proteolytic ferments to activate the precursor.

(2) Trypsin may release a chemical group containing ribonucleic acid and a masking group. If there were unmasked toxin molecules still containing nucleic acid, trypsinization might change the charge by releasing the chemical group containing nucleic acid.

(3) The group containing ribonucleic acid may be a masking group which incompletely covers the toxic groupings. It is the release of this chemical group by trypsin which increases the toxicity of the molecule.

If (2) were true, then two different molecular structures for type E toxin must be postulated. Naturally formed toxin contains nucleic acid and is of acidic nature and the toxin formed by trypsinization contains no nucleic acid and is neutral or weakly basic. If (1) or (2) did not hold, then activation may mean increase of toxicity per molecule, and if either (1) or (2) was proved then activation would mean formation of new molecules of toxin.

In any event, it is reasonable to retain the term "precursor" to refer to an acidic substance from the cells which is changed into a neutral or weakly basic substance associated with toxicity.

From the results obtained, proposed activation processes are schematically diagrammed in figure 9.

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SUMMARY

Bacterial cells from young cultures of *Clostridium botulinum* type E yielded more toxin by activation than the culture supernatant.

Isolation of precursor was accomplished from young bacterial cells by extraction, precipitation with ammonium sulfate, dialysis, and lyophilization.

Partially purified precursor samples were chemically characterized. Ribonucleic acid was found in these samples.

Starch electrophoresis demonstrated that activation of type E "precursor" by trypsin entails a change in charge from negative to neutral or positive, possibly due to the release of ribonucleic acid.

A possible mechanism of activation by trypsin was discussed and the term "precursor" was defined.

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