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Activation of *Clostridium botulinum* Type B Toxin by an Endogenous Enzyme[1]

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It was previously postulated, based on indirect evidence, that *Clostridium botulinum* type B produces neurotoxin which is initially of low toxicity but which then becomes activated to highly toxic form by the action of an endogenous enzyme(s). The first direct in vitro experimental evidence in support of this hypothesis is presented here. The mildly active toxin (progenitor toxin) produced by *C. botulinum* type B (Lamanna) was isolated from the filtrate of a 24-hr culture and partially purified chromatographically. An enzyme that activates the progenitor toxin was also isolated from the filtrate of a 96-hr culture and purified 200-fold. The enzyme hydrolyzes synthetic substrates of trypsin but not of chymotrypsin.

The toxicity of a liquid culture of *Clostridium botulinum* type A or B does not parallel growth of the organisms. The neurotoxin appears in the medium primarily after autolysis. Most of the toxicity in the medium develops in the absence of (i) cell multiplication, (ii) protein synthesis, and (iii) significant increase in total extracellular protein (3, 13). Maximum toxin titer is reached 24 to 72 hr after autolysis of the cells begins (2, 11, 12). Bonventre and Kempe (1) showed that the toxicity of culture filtrates of type A and B organisms withdrawn at 12 hr was enhanced by treatment with trypsin (EC 3.4.4.4). No increase in toxicity of the filtrate from 96-hr cultures resulted from trypsin treatment. This is the time when maximum toxicity developed without any external manipulation of the medium. Based on these and other observations (2), Bonventre and Kempe (3) postulated that botulinum toxin of type A and B cells is produced initially as a "precursor" with low toxicity and is subsequently activated into a highly toxic form by a proteolytic enzyme(s) produced by the organisms. The experiments of Iida (10) agree with this observation.

The term "progenitor" toxin will be used henceforth to refer to the material that is toxic before activation. The term "protoxin" will be used for a nontoxic precursor (C. Lamanna, *personal communication*, and reference 15).

Because the hypothesis of Bonventre and Kempe was based on work with culture filtrates or supernatant solutions, a highly complex mixture, and with trypsin, an enzyme foreign to the culture, more definitive evidence for the validity of the postulated events required isolation from the same culture of a progenitor toxin and an enzyme(s) capable of activating the progenitor toxin. This report presents evidence, for the first time, of activation of partially purified progenitor toxin by a purified enzyme, both isolated from cultures of the same *C. botulinum* type B strain.

MATERIALS AND METHODS

Cultural procedures. *C. botulinum* type B, strain Lamanna, received from D. A. Boroff, was used. Stocks for inoculating media were prepared by growing the culture in a dialysis sac (7) for 96 ± 4 hr at 37 C. After a culture had acquired maximum toxicity [2×10^6 to 3×10^6 minimum lethal doses (MLD)/ml], 2-ml portions of the culture were placed in tubes, frozen in an acetone-dry ice mixture, and stored at −20 C. All experiments were initiated with these frozen cultures. Cultures were grown at 37 C by two methods depending on the product desired (*see below*). The dialysis sac procedure (7) was used to obtain the progenitor toxin activating enzyme (PTA enzyme). The progenitor toxin was produced without the dialysis sac in a medium composed of 2% Proteose peptone no. 2 (Difco), 1% yeast extract (BBL), 1% N-Z-Amine type B (Sheffield Chemical, Norwich, N.Y.), 0.45% NaCl, 0.05% sodium thioglycolate, and 1% glucose. The medium, adjusted to *p*H 7.3 with saturated NaOH, was autoclaved (20 min, 120 C) and while hot was used to fill 500-ml volumetric cylinders (1$^{13}/_{16}$ by 14½ inches) to the 500-ml level. The cylinders were cooled for 1 hr in buckets filled with water at room temperature and then kept in an incubator at 37 C for 3 hr. Two milliliters of

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a frozen culture, thawed at room temperature for 30 min, and 5 ml of sterile filtered glucose solution [1% (w/v)] were added to the 500 ml of medium.

Toxicity assay. Mice were injected intravenously, and time, in minutes, from challenge to death was used as a measure of toxicity in terms of MLD (4). These values were obtained from a standard curve of survival time versus log MLD. The toxicity data are reported below as survival time and MLD/ml. Despite limitations of this assay (16), the conclusions drawn here as to the relative toxicities are considered valid because no statement about the specific toxicity of a sample needs to be made. Presence of the progenitor toxin in a sample was assayed on the basis of an increase in toxicity of the sample after treatment with 125 μg of trypsin (2 times crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) for 2 hr at 37 C, *p*H 6.0. Unless stated otherwise, a reaction mixture consisted of 1.0 ml of test sample, 0.1 ml of 0.2 M phosphate buffer (*p*H 6.0), and 0.1 ml of trypsin dissolved in the same buffer. Control tubes had no trypsin. Chromatographic fractions were dialyzed against 20 volumes of 0.05 M phosphate buffer (*p*H 6.0) for 2 hr at 4 C before treatment with trypsin as described above.

Assays of PTA. For the detection and assay of the PTA enzyme, the progenitor toxin would have been the logical and natural substrate to employ. The limited amount of reasonably pure progenitor toxin plus the uncertainty of its activation by the enzyme led to some other sensitive and reliable assay. Because progenitor toxin was activated by trypsin, it was assumed that the endogenous PTA enzyme might have a trypsin-like property, and therefore a synthetic substrate of trypsin, *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) was selected as a test substrate for the PTA enzyme. The enzyme hydrolyzed BAPNA, and it was assayed at 37 C by a kinetic method (8). A 44.0-mg amount of the reagent (Schwarz BioResearch, Inc., Orangeburg, N.Y.), initially dissolved in 1.0 ml of dimethylsulfoxide (Eastman Organic Chemicals, Rochester, N.Y.), was diluted to 100 ml with 0.04 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.8) containing 5 mM $CaCl_2$ or other buffers. A unit of enzyme activity is defined as 1 μmole of BAPNA hydrolyzed per min (ϵ_M of *p*-nitroaniline at 410 nm = 8,800, see reference 8) in the Tris-hydrochloride buffer. The specific activity is expressed as units per milligram of protein. The amount of enzyme present in the culture during its incubation is expressed as units per milliliter of culture filtrate. The enzyme activity in the different steps during purification was assayed as follows. A 0.1-ml amount of test sample was incubated with 0.1 ml of dithiothreitol (DTT; 0.05 M) for 15 min at room temperature; 3.0 ml of BAPNA was added to the sample, and the absorbance was measured at 410 nm after 5 min of incubation at 37 C. After purification, the PTA enzyme was assayed for (i) its ability to activate progenitor toxin (*see below*), (ii) esterase activity with *p*-toluenesulfonyl-L-arginine methyl ester (TAME) (9), and (iii) chymotrypsin-like activity with *N*-acetyl-L-tyrosine ethyl ester (ATEE) (18). TAME and ATEE were obtained from Worthington Biochemical Corp., Freehold, N.J. The reducing agent DTT was obtained from Calbiochem, Los Angeles, Calif.

Determination of protein. Protein concentration was measured from absorbance at 278 nm, 1.0-cm light path, or was calculated from the absorbance at 260 and 280 nm (20). A spectrophotometer (Hitachi-Perkin Elmer UV-Vis model 139) connected to a digital readout 139 (Arthur H. Thomas Co., Philadelphia, Pa.) was used for all spectrophotometric measurements.

Column chromatography and buffers. The preparation, packing, and operation of diethylaminoethyl (DEAE)-cellulose and gel filtration columns were carried out as previously described (6). All columns were operated at 26 ± 2 C with gravity flow. Buffers were prepared by titrating the acidic and basic conjugates of the same molarity to the desired *p*H. The following buffers were used: 0.067 M citrate-phosphate, *p*H 5.6 (citric acid and Na_2HPO_4); 0.2 M citrate, *p*H 6.0 (citric acid and trisodium citrate); 0.04 M Tris-hydrochloride, *p*H 7.8, containing 5 mM $CaCl_2$ (free base Tris and HCl); and 0.2 M phosphate, *p*H 6.0 (Na_2HPO_4 and NaH_2PO_4).

RESULTS

The presence of PTA enzyme and progenitor toxin in a culture and enhancement of its toxicity as a function of age of the culture was studied by growing cells, without the dialysis sac, as described above. Samples (2 ml) withdrawn at intervals up to 7 days were stored at −20 C. These samples were thawed together at room temperature and centrifuged to remove cells and debris. The supernatant solutions were filtered through a 1.2 μm filter (Millipore Corp.) and assayed for the PTA enzyme and toxicity with or without trypsin treatment. Results are shown in Table 1. The toxin titer, after attaining a maximum at 89 hr, began to fall. As the culture aged, its toxicity (nontrypsinized) increased, but degree of activation (i.e., increase in toxicity due to trypsin) diminished. Maximum activation was found in the 24-hr culture. Appearance of the enzyme between 24 and 39 hr coincided with a 93-fold increase in toxicity (survival time 156 min to 58 min, or 1.5×10^3 MLD per ml to 1.4×10^5 MLD per ml). During the next 50 hr, while the enzyme level was constant, toxicity increased another 6.4-fold (survival time, 58 min to 37 min, or 1.4×10^5 MLD per ml to 9×10^5 MLD/ml), giving a total of about a 600-fold rise in toxicity, occurring naturally, between 24 and 89 hr.

Purification of progenitor toxin. For harvesting the progenitor toxin, a 24-hr culture was selected. The culture was centrifuged, filtered, and subjected to $(NH_4)_2SO_4$ precipitation as described elsewhere (5). After 48 hr at 4 C, the precipitate was collected by centrifugation at 27,000 × *g* for 20 min. The sediment was suspended in *p*H 5.6 buffer in a volume equal to 0.5% of the culture filtrate volume. This material, referred to as crude progenitor toxin, remained stable for at least 8 weeks stored at −20

TABLE 1. *Change in level of progenitor toxin-activating enzyme and toxicity of a culture during incubation*

Age of culture (hr)	Enzyme activity (unit/ml)	Toxicity[a]		Increase in toxicity (MLD × 10^3/ml)
		Without trypsin (A)	With trypsin (B)	A/B
17	0.010	120[b]	108	6/3.6 (1.6-fold)[c]
24	0.022	156	100	9/1.5 (6.0-fold)
30	0.180	100	75	37/9 (4.1-fold)
39	0.254	58	45	440/140 (3.1-fold)
48	0.256	55		
63	0.248	51		
72	0.254	44		
89	0.249	37	38	840/900 (0.93-fold) (not significant)
96	0.253	44		
144	0.247	48		
168	0.262	56		

[a] Expressed as survival time in min, mean of four determinations.

[b] Higher toxicity found at 17 hr than at 24 hr was most likely due to 2 ml of seed culture (2 × 10^6 to 3 × 10^6 MLD/ml) used to inoculate 500 ml of medium. The resultant initial toxicity was probably inactivated with time due to near alkaline *p*H of the medium.

[c] The low activation apparently represents activation of a small amount of progenitor toxin released by the growing organisms and the toxin introduced with the inoculum which was nearly fully activated.

C. The crude progenitor toxin was dialyzed at 4 C against *p*H 5.6 buffer and centrifuged according to procedures described before (5). The supernatant solution in 5.0-ml portions was applied to DEAE-cellulose columns (1 by 30 cm) equilibrated with *p*H 5.6 buffer. The columns were eluted with this buffer. A large, sharp peak emerged (Fig. 1). Fractions across the peak with absorbance of 0.075 and above were pooled. A large amount of pigment and essentially all nucleic acid (measured by absorbance at 260 and 280 nm) were retained by the column.

Solid $(NH_4)_2SO_4$ was added (39 g/100 ml) to the pooled fractions at room temperature. After 48 hr at 4 C, the resulting precipitate was collected by centrifugation at 39,000 × *g* for 20 min. The sediment was suspended in *p*H 5.6 buffer in a volume equal to 10% of the total volume of the pooled fractions. The suspension was left at room temperature for 30 min with occasional gentle trituration with a glass rod. The insoluble material was removed by centrifugation at 12,000 × *g* for 15 min. The supernatant solution in 5.0-ml portions was applied to a Sephadex G-100 column (2.2 by 45 cm). The column was equilibrated and eluted with *p*H 5.6 buffer. The material applied to the column emerged as three peaks (Fig. 2). Toxicity of the material in the first peak, eluted essentially at the void volume of the column, could be increased sevenfold (survival time 61 min to 39 min, or 1.1 × 10^5 MLD/ml to 7.6 × 10^5

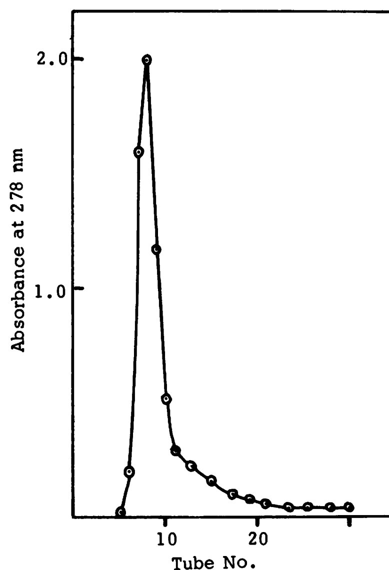

FIG. 1. *Chromatography of crude progenitor toxin on a diethylaminoethyl-cellulose column. Fractions (3 ml/tube) were collected at a flow rate of 35 to 40 ml/hr.*

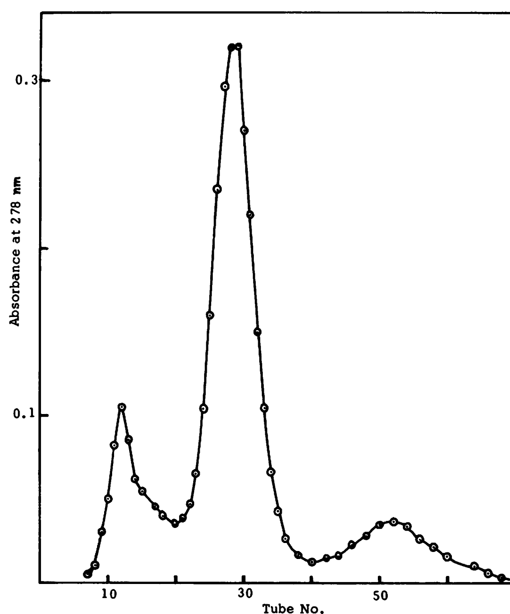

FIG. 2. *Gel filtration of progenitor toxin on a Sephadex G-100 column. Fractions (3 ml/tube) were collected at a flow rate of 35 to 40 ml/hr. Fractions 12, 29, and 52 were assayed for activation.*

MLD/ml) with trypsin treatment. This material will be referred to as G-100 progenitor toxin. The material in the second and third peaks contained trace amounts of toxicity. Attempts to enhance toxicity of these samples with trypsin gave ambiguous results. The G-100 progenitor toxin was not purified further (*see below*).

Purification of PTA enzyme. A 96-hr culture was centrifuged at 14,000 × *g* for 40 min. The supernatant solution was passed through a 1.2 μm filter (Millipore). Solid $(NH_4)_2SO_4$ was added (47.2 g/100 ml) to 770 ml of the filtrate solution at room temperature. The filtrate solution had 283 units of enzyme activity and a specific activity of 0.024 unit/mg. After 48 hr at 4 C, the precipitate was collected by centrifugation at 27,000 × *g* for 20 min. The sediment was suspended in *p*H 7.8 buffer in a volume equal to 3% of the original volume of the culture filtrate. A 23-ml portion of the suspension was dialyzed against 230 ml of *p*H 7.8 buffer at room temperature for 4 hr. During dialysis the buffer was changed hourly. The dialyzed sample was centrifuged at 12,000 × *g* for 10 min. The supernatant solution, with a specific activity of 0.52 unit/mg and 203 units of enzyme activity (i.e., 71% recovery), was applied on a DEAE-cellulose column (2 by 20 cm) equilibrated with *p*H 7.8 buffer. After the column was washed extensively with the equilibrating buffer, a linear gradient of increasing NaCl concentration was applied. Figure 3 shows the elution pattern. The enzyme activity was confined to a narrow region of fractions eluted with increasing concentration of Cl^- in the buffer. Fractions which had at least 25% of the enzyme activity present in the peak tube were pooled. The pooled material had a specific activity of 1.1 units/mg and a total of 173 units of enzyme activity (i.e., 61% recovery).

The pooled material was applied on a Sephadex G-100 column (2.2 by 50 cm) in 10-ml portions. The column was equilibrated and eluted with *p*H 7.8 buffer. Three peaks emerged from the column (Fig. 4). Most of the enzyme activity was associated with the second peak. A number of fractions with enzyme activity (see legend of Fig. 4) were pooled. The pooled material had a specific activity of 4.8 units/mg and a total of 96 units of enzyme activity (i.e., 33% recovery).

A total of 94 ml of the pooled material, obtained from several gel filtration runs, was applied on a DEAE-cellulose column (0.9 by 14 cm) equilibrated with *p*H 7.8 buffer. The column was washed with the same buffer and eluted with a linear gradient of increasing NaCl concentration. The elution profile presented in Fig. 5 shows only one peak. The enzyme activity was essentially coincident with the protein profile of this peak which contained a total of 35 units of enzyme activity (i.e., 12% recovery). Fractions 88 to 92 had similar specific activities that averaged 5.13 units/mg. This specific activity represents about 213-fold purification of the enzyme beginning with the culture filtrate. These fractions had no demonstrable toxicity. These and similar fractions were used as purified PTA enzyme in experiments described below.

Effect of PTA enzyme on progenitor toxin. The

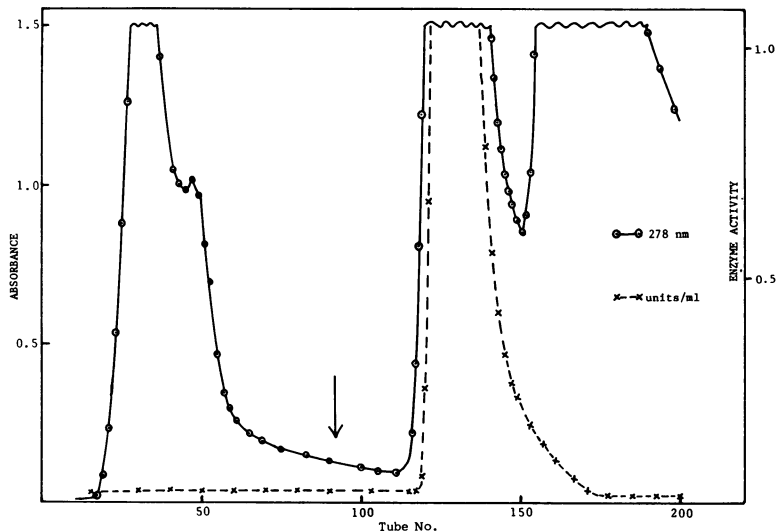

Fig. 3. *Chromatography of crude progenitor toxin activating enzyme on a diethylaminoethyl-cellulose column. A linear gradient (130 ml of pH 7.8 buffer plus 130 ml of pH 7.8 buffer containing 1.0* M *NaCl) elution was started at the position marked with arrow. Fractions (3 ml/tube) were collected at a flow rate of 20 to 25 ml/hr.*

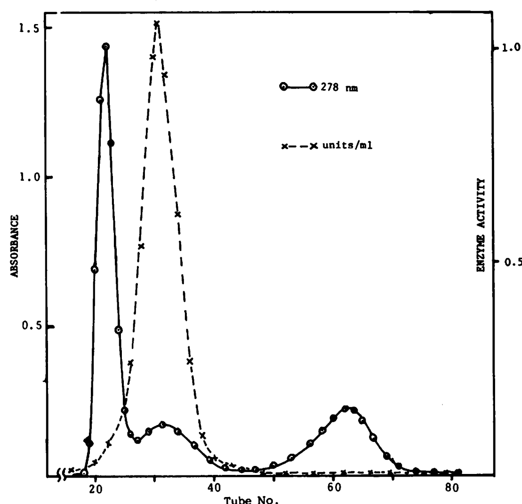

FIG. 4. *Gel filtration of progenitor toxin activating enzyme on a Sephadex G-100 column. Fractions (3 ml/tube) were collected at a flow rate of 35 to 40 ml/hr. Fractions 28 to 37 were pooled for rechromatography.*

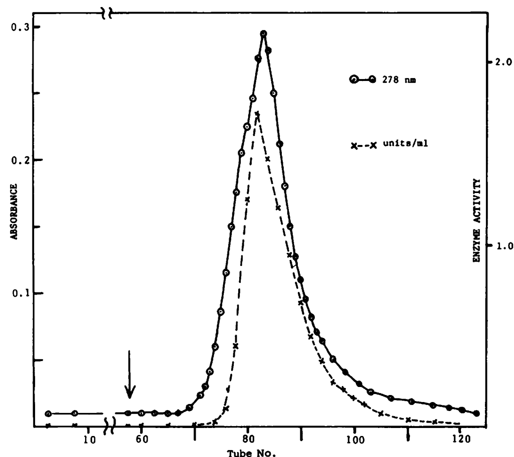

FIG. 5. *Rechromatography of progenitor toxin activating enzyme on a diethylaminoethyl-cellulose column. A linear gradient (100 ml of pH 7.8 buffer plus 100 ml of pH 7.8 buffer containing 0.3 M NaCl) was started at tube number 58 as marked by arrow. Fractions (2 ml/tube) were collected at a flow rate of 20 ml/hr.*

toxicity of progenitor toxin after treatment with the PTA enzyme at pH 6.0 for 2 hr at 37 C was significantly enhanced. This was demonstrated with crude and purified progenitor toxin (Table 2). No attempt was made to obtain enhanced activation by the PTA enzyme beyond that observed here by varying the reaction conditions.

Effect of PTA enzyme on synthetic substrates. The PTA enzyme hydrolyzed BAPNA and

TABLE 2. *Activation of progenitor toxin by PTA enzyme*

Reaction	Toxicity		Degree of activation (from control)
	Survival time (min)	MLD/ml (mean of 4 determinations)	
Crude progenitor toxin[a] + buffer (control)[b]	108	6×10^{3}	
Crude progenitor toxin + PTA enzyme[b]	77	3.4×10^{4}	5.7
G-100 progenitor toxin + buffer (control)[c]	59	1.3×10^{6}	
G-100 progenitor toxin + PTA enzyme[c]	47	3.7×10^{6}	2.8

[a] Crude progenitor toxin was dialyzed at 4 C for 2 hr against 30 volumes of 0.2 M citrate buffer (pH 6.0) before use. The progenitor toxin activating (PTA) enzyme had an absorbance of 0.327 at 278 nm and a specific activity of 4.9 units/mg. The absorbance of G-100 progenitor toxin was 0.245 at 278 nm and had a ratio of 260 nm/278 nm = 0.62.

[b] After incubating 0.1 ml of dithiolthreitol (DTT) (0.05 M) with 0.5 ml of 0.2 M citrate buffer (pH 6.0) or 0.5 ml of PTA enzyme for 30 min at 4 C, 0.3 ml of crude progenitor toxin was added to each tube. Enough citrate buffer was added in each to make the final volume 2.4 ml.

[c] After incubating 0.1 ml of DTT (0.05 M) with 0.5 ml of pH 7.8 buffer or 0.5 ml of PTA enzyme for 30 min at 4 C, 0.5 ml of G-100 progenitor toxin was added to each tube to make the final volume 1.1 ml.

TAME but not ATEE. Figure 6 shows that the velocity of hydrolysis of BAPNA at three different enzyme concentrations was linear up to 7 min under the conditions employed. An increase in enzyme concentration increased the concentration of the reaction product proportionately. Figure 7 shows that the rate of hydrolysis of TAME, proportional to four different concentrations of the enzyme, was linear up to at least 8 min. Hydrolysis of ATEE by the PTA enzyme did not occur as measured by changes in absorbance at 237 nm. The concentration of enzyme used for ATEE was higher than that used in experiments with BAPNA and was equal to the highest used with TAME. Under the conditions employed, ATEE was hydrolyzed by chymotrypsin (EC 3.4.4.5).

Optimal pH of PTA enzyme activity. The optimal pH of the enzyme for hydrolysis of BAPNA was found to be 6.0. A constant amount of enzyme, after incubation in 0.005 M DTT at 4 C for 1 hr, was assayed in 0.06 M phosphate buffers of pH ranging from 5.8 to 8.0 in increments of 0.2 units.

DISCUSSION

The greater enhancement of toxicity by trypsin of supernatant solutions from young cultures compared with what was found in old cultures

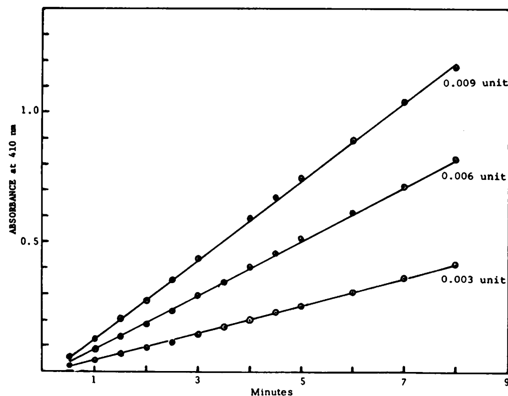

FIG. 6. *Amidase activity of progenitor toxin activating enzyme. To 0.3, 0.2, and 0.1 ml of enzyme (absorbance of 0.15 at 278 nm and specific activity of 5.1 units/mg) were added 0.1 ml of dithiothreitol (0.05 M) and enough pH 7.6 buffer to make a final volume of 0.5 ml. These diluted solutions of the enzyme were incubated at 4 C for 1 hr. To portions (0.1 ml) of these solutions, 3.0 ml of N-benzoyl-DL-arginine-p-nitroanilide was added, and the reaction at 37 C was followed by recording absorbance at 410 nm. Final concentrations of the enzyme protein added in the reaction mixtures were 0.009, 0.006, and 0.003 units of absorbance at 278 nm.*

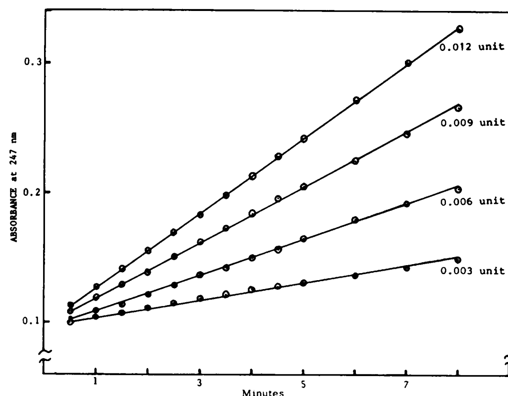

FIG. 7. *Esterase activity of progenitor toxin activating enzyme. To 0.1-ml portions of diluted enzyme solutions (see Fig. 6) was added 2.9 ml of p-toluenesulfonyl-L-arginine methyl ester (0.87 × 10^{-3} M in 0.05 M phosphate buffer, pH 8.0), and the reaction at 25 C was followed by recording absorbance at 247 nm. Final concentrations of enzyme protein added in the reaction mixtures were 0.012, 0.009, 0.006, and 0.003 units of absorbance at 278 nm.*

confirmed the observations of Bonventre and Kempe (1) and Iida (10). Consideration of the appearance of the PTA enzyme activity as a function of the age and rise in toxicity of the culture together with activation of the partially purified progenitor toxin by the highly purified enzyme provided evidence closer to the natural situation of toxin formation in a pure culture as was proposed by Bonventre and Kempe (3). Although, as shown here and reported elsewhere (2, 10, 12), many hours are required to attain maximum toxicity and, therefore, maximum activation under natural cultural conditions, the in vitro activation results reported here are based on 2 hr of reaction. This report does not imply that natural activation of progenitor toxin occurs because of the PTA enzyme only. Such a conclusion must wait until all the proteases present in the culture are tested singly and in combination on the progenitor toxin or a suitable mutant is found that produces progenitor toxin but does not activate it in the culture in the absence of PTA enzyme production.

The rationale for using two sets of conditions, one for PTA enzyme and the other for progenitor toxin, was to make purification of the two easier. Examination of the data in Table 1 shows that maximal concentrations of the progenitor toxin and the enzyme could be harvested at different times, e.g., at 24 hr and at 39 hr or later, respectively. The enzyme and the progenitor toxin could be isolated from cultures grown in the same way, but purification of the enzyme was easier when the culture was 96 hr old and grown in a dialysis sac. The purified enzyme shows trypsin-like substrate specificity. The amide and ester substrates, BAPNA and TAME, both have an arginyl moiety and are substrates of trypsin. When arginine was replaced with a tyrosyl moiety, as in ATEE, the substrate was not hydrolyzed. Proteolytic activity of the enzyme on peptides of various amino acid residues is under study. Although proteolytic enzymes of *C. botulinum* have been detected, based on gelatin liquefication or casein hydrolysis (3, 10), there is no previous documentation of the PTA enzyme in the culture of *C. botulinum* type B. An aminopeptidase, probably of the class EC 3.4.1.1, isolated from cultures of the same organisms (17) differs from the PTA enzyme. The aminopeptidase requires for activity a free α-amino group but not a basic amino acid in the substrate. The PTA enzyme apparently requires substrates with basic amino acids where the α-amino group may be blocked.

The G-100 progenitor toxin is partially purified. This material was resolved into two peaks on a Sephadex G-200 column at *p*H 7.2 (results not shown). Of the two peaks, the faster moving one was nontoxic, whereas the second peak was toxic. Separation of the two components caused a loss in the potential to gain toxicity as a result of trypsin treatment. A similar inactivation of type E "precursor toxin" (actually progenitor toxin) was noted when the preparation was exposed to *p*H 7 or 8 and resolved into two compo-

nents (14). Conditions have not yet been found for purifying the progenitor toxin beyond the step of gel filtration on a Sephadex G-100 column, which allows retention of activity. It remains to be shown whether the low level of toxicity of the progenitor toxin is due to an intrinsic toxicity of the molecule or due to contamination with the endogenously activated toxin. Except for an increase in the specific toxicity due to activation, the progenitor toxin of type B, like that of type E (14), cannot yet be distinguished from the corresponding activated toxin. Activation of type E toxin results in appearance of more toxigenic sites on the molecule without a detectable change in its antigenic character (14, 19). Apparently the structural change(s) associated with activation of type B or E toxin is small enough to elude standard separation and identification methods. Detection of fine structural changes by means of determination of N-terminal amino acid, change in optical rotatory dispersion, absorption and fluorescence spectra, etc., is feasible only after the progenitor toxin is obtained in a homogenously pure state. Attainment of such a pure preparation retaining its biological activity is a goal yet to be achieved.

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