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Molecular Forms of Neurotoxins in Proteolytic *Clostridium botulinum* Type B Cultures

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A modified purification method was used to isolate the neurotoxin of proteolytic *Clostridium botulinum* type B strain Lamanna. The preparation was found to be a mixture of two protein forms. They were of molecular weight 152,000 and could not be separated by ion-exchange chromatography or electrophoresis in polyacrylamide gel. One was a single polypeptide chain, and the other was a dichain molecule (nicked toxin) held together by an interchain disulfide bond(s). Trypsinization increased the toxicity of the toxin preparation and converted the single-chain molecules into dichain forms that were indistinguishable from the endogenously generated nicked toxin. A protease of the type B culture, with substrate specificity similar to that of trypsin, did not change detectably the molecular form of unnicked type E toxin, although toxicity was increased. Higher toxicity was obtained when unnicked type E was trypsinized; the resulting preparation contained only nicked toxin molecules.

Neurotoxins of *Clostridium botulinum* are synthesized as molecules having low or possibly no toxicity. Their characteristically high specific toxicity is attained when these "native" toxins are activated. With proteolytic cultures (types A and B), activation occurs endogenously during toxin production from the action of protease(s) produced by the organisms (10, 11); in cases of nonproteolytic strains (types B, E, and F), the full activation is obtained when the toxin is trypsinized experimentally (8, 10). Trypsin (EC 3.4.4.4) does not increase the toxicity of type A toxin isolated from cultures incubated for maximum toxicity, but gives high activation of type E toxin (10). Toxin of proteolytic type B strain Lamanna is intermediate in that its trypsinization results in a low but detectable toxicity increase (10).

The fully activated botulinum toxin types studied have a common "nicked" protein structure that is comparable to that described for diphtheria toxin (2, 13). Nicked botulinum toxins as defined here are the toxic molecules of molecular weight 145,000 to 167,000 which are composed of a heavy (H) and a light (L) chain held together by a disulfide bond(s). H is approximately twice the molecular weight of L (1, 5, 12, 14). The untrypsinized type E toxin has the molecular weight of the activated form, but is a single-chain unnicked protein which, during its activation by trypsin, is nicked to become a two-chain protein. This dichain molecule is comparable to the endogenously activated toxin of proteolytic cultures. The H and L chains of nicked toxins can be separated by polyacrylamide sodium dodecyl sulfate electrophoresis only after their disulfides are reduced (5).

The work reported here relates the molecular structure of the toxin purified from the proteolytic type B Lamanna strain to its low activatability with trypsin. Possible other molecular changes of native toxin during its endogenous activation are considered.

MATERIALS AND METHODS

Buffers were prepared by titrating acidic and basic components of the same molarity; pH values are those determined at 23°C. Citrate buffer components were citric acid and trisodium citrate; phosphate buffer was made with di- and monosodium phosphate and acetate buffer was made with acetic acid and sodium acetate; and Tris-hydrochloride was tris(hydroxymethyl)aminomethane and HCl. Sephadex materials (Pharmacia Fine Chemicals) were of 40- to 120-μm beads, and chromatographic steps were by gravity flow at room temperature except where noted. Protein concentrations were determined as absorbances at 278 nm (A_{278}). Trypsin, freed of chymotryptic activity with L-tosylamido-2-phenylethylchloromethyl ketone, and soybean trypsin inhibitor were from Worthington Biochemical Corp., Freehold, N.J. Toxicity was assayed as 50% lethal dose (LD_{50}) for mice (7).

Toxin preparations were trypsinized by incubation at 37°C with enzyme dissolved in 0.01 or 0.02 M phosphate buffer (pH 6.0). After incubation, a 0.1-ml aliquot was mixed with soybean trypsin inhibitor at a trypsin/inhibitor ratio of 1:1.5 (wt/wt). A second aliquot, added to a solution made of 20 μl of 10%

dodecyl sulfate and 0.1 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1% dodecyl sulfate and 3 μl of mercaptoethanol, was immediately placed in a boiling water bath and heated for 5 min.

Polyacrylamide gel electrophoresis with dodecyl sulfate (PAGE) was that used previously (5). Relative concentrations of unnicked toxin, H and L chains, in a solution were estimated, after their separation by PAGE, from densitometric tracings made of the stained gels at 560 nm with a Gilford linear transport scanner (model 2410S) running at 2 cm/min and with recorder chart speed of 5.01 mm/min. That the areas in the traced peaks reflected protein concentrations was shown by a test in which separate gels were charged with 100, 80, 60, 40, 20, or 10 μl of a type B toxin preparation and electrophoresed for 6 h. For each molecular species, the plot of areas against sample volume showed a straight-line relationship (Fig. 1). The H chain was more chromogenic than the L chain, so that, within the precision of the procedure, H/L A_{560} ratios of 1.3:1 to 1.6:1 represented equimolar amounts of the two proteins.

Type E neurotoxin preparation (7) showed only the unnicked toxin band (molecular weight, 147,000) in PAGE. Toxicity after trypsinization was 1.71×10^7 LD_{50} per 1.0 A_{278}.

The protease (TLE) with substrate specificity similar to that of trypsin was an essentially homogeneous preparation that was derived from the Lamanna culture strain used as the source of type B toxin (6). Before use, it was reduced for 1 h at 37°C with 5 mM dithiothreitol solution made in 0.05 M acetate buffer (pH 6.0) containing 2 mM $CaCl_2$.

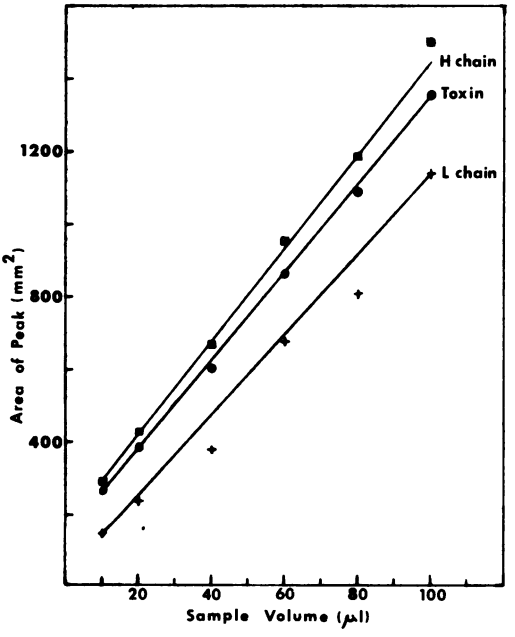

FIG. 1. *Relationship between protein amounts in bands separated by PAGE and the areas of A_{560} densitometric peaks of stained bands. Samples are different volumes of a type B toxin (A_{278} = 0.146) reduced with mercaptoethanol; H and L chains are in a 1:1 molar ratio in all samples.*

Type B neurotoxin was purified from 8-liter culture lots grown in carboys of 9-liter capacity. Culture medium for producing progenitor toxin (3), without added NaCl, was inoculated with 2 ml of culture stock that had been stored at −20°C. Incubation for toxin production was 96 h at 37°C or 168 h at 30°C. Toxin purification was by a procedure modified from the one used previously (4).

Crude toxic precipitate was obtained by dissolving 313 g of $(NH_4)_2SO_4$ per liter of incubated culture and holding it for 48 h at 4°C. The precipitate was collected by centrifugation and extracted twice with 100 ml of 0.05 M citrate buffer (pH 5.5). The precipitate, obtained by dissolving 351 g of $(NH_4)_2SO_4$ per liter of clarified (by centrifugation) pool of the extracts, was dissolved in 40 ml of citrate buffer. After dialyzing against the same buffer, a 20-ml sample (A_{278} = 25.6 and A_{260} = 37 to A_{278} = 80 and A_{260} = 124) was loaded on a diethylaminoethyl (DEAE)-Sephadex A-50 column (2.2 by 45 cm). Essentially all the applied toxicity was recovered in the first protein peak that was eluted in washing the column with the citrate buffer. The A_{260}/A_{278} ratio of 0.5 to 0.55 in the toxic fractions indicated removal of most of the nucleic acids.

Disodium ethylenediaminetetraacetate (EDTA) and $(NH_4)_2SO_4$ were dissolved in the pool of the toxic fractions to 5 mM and 351 g/liter, respectively. The precipitate, collected after 48 h at 4°C, was dissolved in 5.0 ml of 0.15 M Tris-hydrochloride buffer (pH 7.4) containing 5 mM EDTA. No more than 1.6 ml of the solution was applied on a Sephadex G-200 column (2.5 by 95 cm) equilibrated with the same buffer. The toxin, which was recovered in the second protein peak, was concentrated with $(NH_4)_2SO_4$ (351 g/liter) and rechromatographed on Sephadex G-200 without EDTA in the buffer.

All subsequent steps were performed at 4°C. The cooled toxic pool was applied to a DEAE-Sephadex A-50 column (1.5 by 25 cm) equilibrated with the Tris-hydrochloride buffer. The major protein peak eluted with a NaCl gradient (150 ml of Tris-hydrochloride buffer + equivolume buffer with 0.15 M NaCl) contained most of the toxin; a small second peak that developed in some runs was discarded. The toxic pool was dialyzed against 0.02 M phosphate buffer (pH 5.95) and then chromatographed on a sulfoethyl (SE)-Sephadex C-50 column to recover neurotoxin in a sharp, symmetrical peak (Fig. 2). The pool of toxic fractions was stored at 4°C in the presence of 5 mM EDTA and 3 mM NaN_3. To process an 8-liter culture lot required sequentially four DEAE-Sephadex A-50, four Sephadex G-200, two repeat Sephadex G-200, one DEAE-Sephadex A-50, and one SE-Sephadex C-50 columns.

RESULTS

The type B toxin purification procedure yielded uniform toxin preparations more consistently than the one used previously (4). About 5 to 7% of the starting toxicity was re-

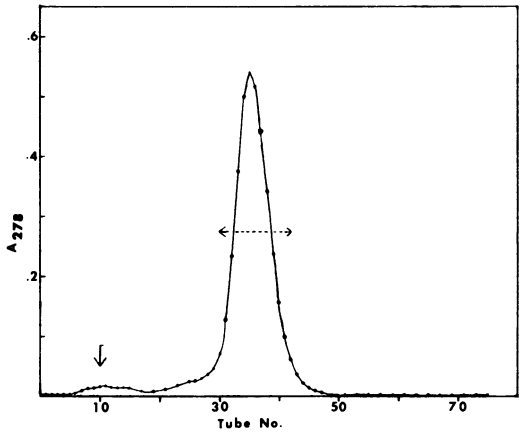

FIG. 2. *Final chromatographic step in purification of type B toxin. A SE-Sephadex C-50 column (1.5 by 25 cm) equilibrated with 0.02 M phosphate buffer (pH 5.95) was loaded with 56 ml of dialyzed solution of A_{278} = 0.38. Gradient elution (150 ml of the pH 5.95 buffer + 150 ml of same buffer containing 0.15 M NaCl) started at arrow. Fractions (4.8 ml/tube) 30 to 42 pooled as purified toxin.*

covered such that 7 to 9 mg of purified toxin (A_{260}/A_{278} = 0.48) was obtained from 8-liter cultures incubated at 37°C for 96 h. Specific toxicity of the preparations (trypsinized) ranged from 4.7×10^7 to 6.2×10^7 mouse LD_{50}/1.0 A_{278}, or 1.14×10^8 LD_{50}/mg of toxin based on $E^{1\%}_{1\,cm}$ of 18.5 (1). The molecular weights of three separately purified type B neurotoxin preparations determined by seven PAGE experiments (5) appeared to be similar. This value of 152,000 was lower than the previously reported values of 165,000, 167,000, and 170,000 (1, 4, 12). The molecular weight of the H chain was essentially twice that of the L chain.

PAGE analyses of toxin preparations not treated with a disulfide reducing agent showed essentially only the expected single band (proteins of molecular weight 152,000), but traces of two other bands could occasionally be detected (Fig. 3A). That these faint, fast-moving bands probably represent the H and L chains, which are normally held together as nicked toxin, was indicated by results of electrophoresing untrypsinized but reduced toxin. The test resolved the same three bands, except that the two faster-moving ones were now much more prominent; the H and L chains, made separable by the reductive treatment, moved coincidently with the original trace bands so that the latter could not be distinguished from the known H and L bands (Fig. 3A,B).

The tests showed the toxin preparations to be essentially a mixture of nicked toxin and unnicked protein of molecular weight 152,000 (Fig. 3A,B; Fig. 4A,B). The unnicked protein was identified as unnicked toxin by the correlated, progressive disappearance of its band with increases of H and L chains during trypsinization of toxin samples. Since this change in electrophoretic pattern could be shown only when the trypsinized sample was reduced (Fig. 3C,D; Fig. 4C), trypsin must have cleaved a bond of a protein, but the cleavage products remained together until the connecting S-S linkage(s) was broken. The behavior was that expected of unnicked toxin. The toxin purified from the proteolytic type B culture was, therefore, a mixture of nicked and unnicked toxin.

The ratio of nicked (sum of H and L chains obtained by PAGE of reduced but untrypsinized preparations) to unnicked toxins in the purified toxin preparations varied with the incubations used for toxin production. In samples purified from cultures incubated at 37°C for 96 h, the ratio ranged from 1:0.4 to 1:0.6; in samples derived from cultures incubated at 30°C for 168 h, the ratio ranged from 1:1.6 to 1:1.8. Trypsinizing these toxin preparations for maximum toxicity increased specific toxicity only two- to threefold.

Type B toxin samples from the two differently incubated cultures were trypsinized at ratios of substrate to enzyme of 20:1, 14.7:1, 9.8:1, 4.9:1, and 3.25:1 (wt/wt) and incubation times of 0, 10, 20, 40, and 60 min. Comparisons of protein concentration in the bands resolved by PAGE showed that L and H chains increased in a fixed (1:1) molar ratio (Fig. 3B–D; Fig. 4B,C).

Possible differences in action of TLE and trypsin on unnicked type E toxin were studied. Toxin (A_{278} = 0.113) was incubated separately with an equal volume of TLE (A_{278} = 0.487) or trypsin (final concentration, 28 μg/ml), both in 0.05 M acetate buffer (pH 6.0) with 2 mM $CaCl_2$. Control was toxin mixed with buffer. After 1 h at 37°C, the mixtures were analyzed for toxicity and by PAGE. The same tests were made with a portion of the TLE-treated sample that was further incubated for 1 h with trypsin at a final concentration of 28 μg/ml.

Toxicities, corrected for dilutions, of toxin treated with TLE, trypsin, and TLE plus trypsin were, respectively, 4, 29, and 8 times (see reference 7 for explanation on such low, high, and intermediate values) higher than the 5.6×10^3 LD_{50}/ml of the control. All samples analyzed by PAGE without reduction gave only the band representative of molecules of 147,000 molecular weight. Reductive treatment of the control and the TLE-treated samples did not change the electrophoretic pattern. When toxin was reduced after being treated with trypsin alone

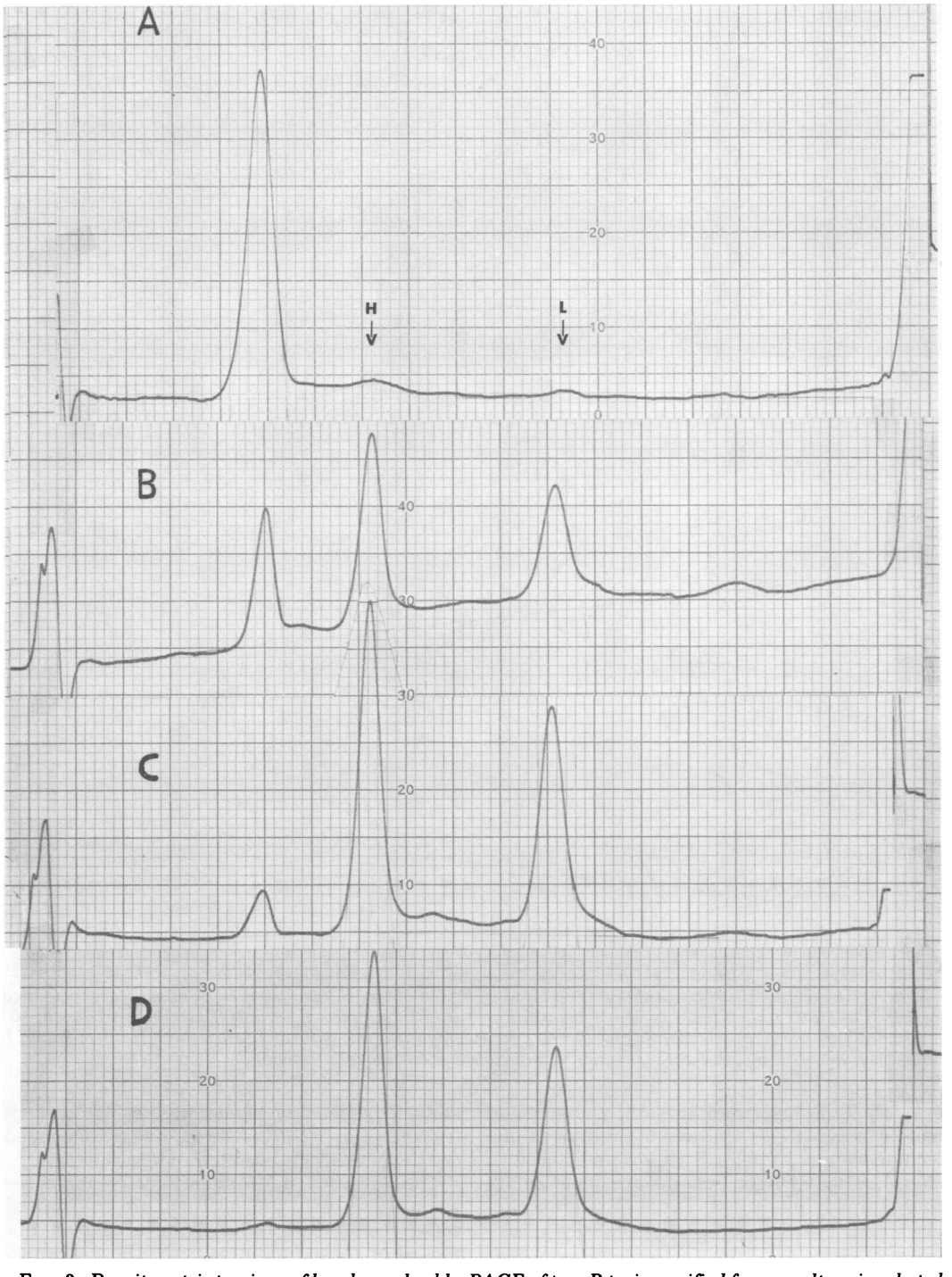

FIG. 3. *Densitometric tracings of bands resolved by PAGE of type B toxin purified from a culture incubated for 96 h at 37°C. A_{278} of toxin before trypsinization = 0.142. Sample on gel is 100 μl. (A) Unreduced; (B) reduced; (C) reduced after trypsinization for 10 min at a toxin/enzyme ratio of 4.9:1 (wt/wt); (D) same as C except trypsinized for 60 min. Ratios of H and L peak areas in B, C, and D are, respectively, 1.2:1, 1.3:1, and 1.3:1. Direction of migration is left to right (anode).*

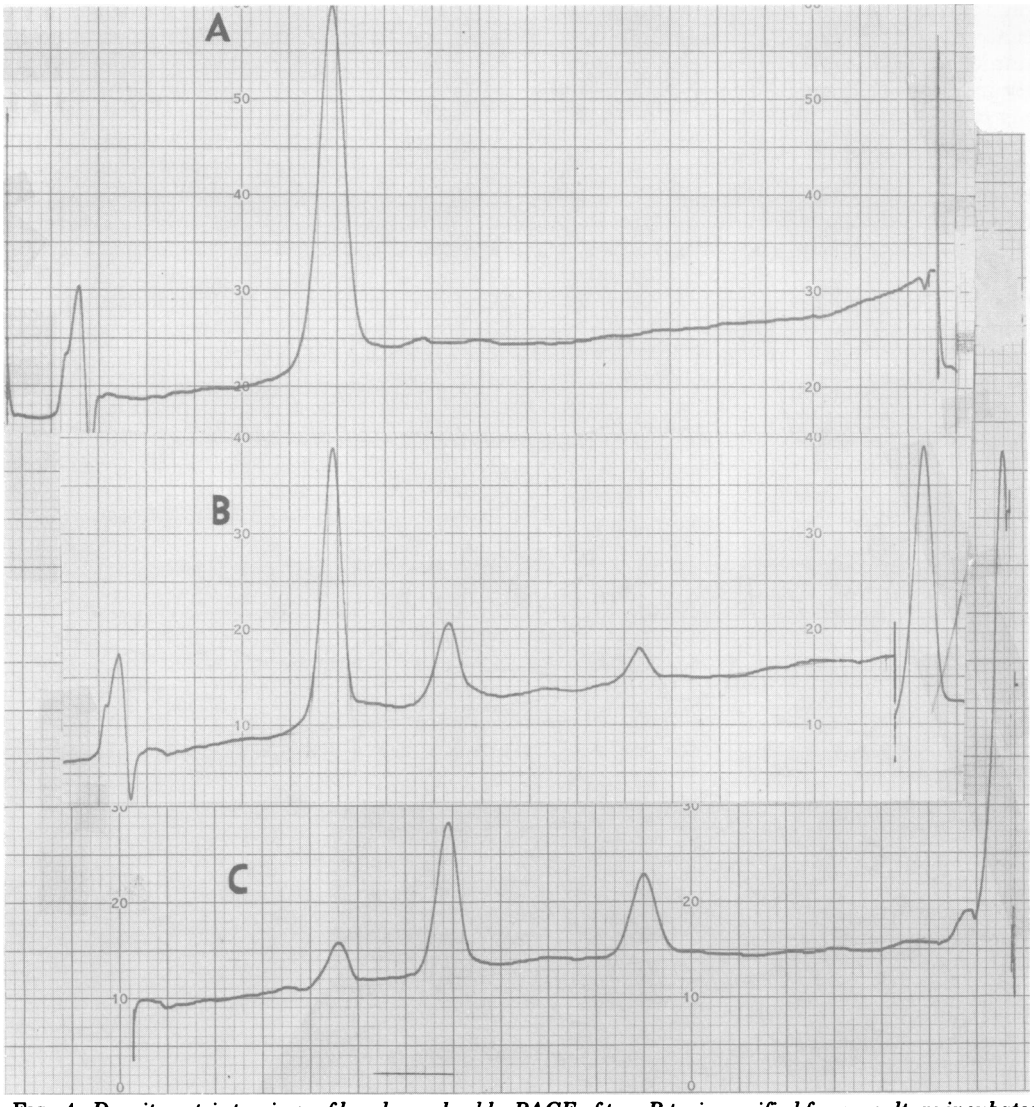

FIG. 4. *Densitometric tracings of bands resolved by PAGE of type B toxin purified from a culture incubated for 168 h at 30°C. A_{278} of toxin before trypsinization = 0.125. Sample on gel is 100 μl. (A) Unreduced; (B) reduced; (C) reduced after trypsinization for 60 min at a toxin/enzyme ratio of 4.9:1 (wt/wt). Ratio of H and L peaks in B and C are both 1.6:1. Direction of migration is left to right (anode).*

or with TLE and then trypsin, the original band was replaced wth those of proteins of molecular weight 102,000 (H chain) and 50,000 (L chain). These PAGE results are identical to those already published (Fig. 1 of reference 5).

DISCUSSION

Excluding the trace amounts of H and L chains that are apparently held together, by noncovalent bonds after reduction of their interchain S-S bond(s), type B toxin purified from a proteolytic culture is a mixture of unnicked and endogenously nicked proteins. The concentration ratios of the two forms of toxin depended on the incubations used to produce the toxin.

The low but detectable toxicity increases when these toxin preparations are trypsinized could result from the enzyme acting on unnicked or nicked toxin or both. Data obtained with other botulinum toxin types suggest the action to be on the unnicked form. There is no toxicity increase upon trypsinizing type A (unpublished data) and F (K. H. Yang, Ph.D. thesis, Univ. of Wisconsin, Madison, 1974) toxin

preparations that are free of unnicked toxin. In contrast, type E preparations containing only unnicked toxin show significant specific toxicity increases that are coincident with the nicking of the molecule by trypsin (5).

Trypsinization of the single-polypeptide-chain form of the neurotoxin may cleave more then one bond, with at least one leading to the formation of the dichain molecule, but splitting of more than one bond may be associated with activation. It is not yet known whether all of the toxicity increase upon trypsinization is from nicking, i.e., the peptide bond cleavage creating the H and L subunits, but the maximum threefold toxicity increase observed after trypsin treatment of the type B preparations is suggestive. These preparations have 1.8:1 to 0.4:1 ratios of unnicked to endogenously nicked toxin. Assuming that nicked toxins, formed endogenously or by trypsinization, are at the highest attainable specific toxicity, it can be calculated that even with a 2:1 ratio of unnicked to nicked toxin forms, trypsinization would not increase toxicity more than threefold.

The H chain of endogenously nicked type B toxin has a molecular weight essentially twice that of the L chain. Action of trypsin on the unnicked toxin form in the purified toxin preparations generates nicked toxin whose constituents are 2:1 in relative molecular sizes. However, these observations do not prove identity of the nicked toxins that are formed endogenously and by trypsinization; cleavage of unnicked toxin either one-third or two-thirds the distance from the C- to N-terminals would both give chains of the observed ratio.

The probability is that the nicked toxins being compared are similar molecules that result from cleavage of the same or very close-by peptide bond(s). If such were not the case, the endogenously formed H chain would have near its center the bond susceptible to trypsin; its cleavage would create two pieces, each of size comparable to the L chain. The situation would be such that trypsinization of the purified toxin, which is a mixture of unnicked and endogenously nicked molecules, would result in more molecules of L-chain dimension than of H chain. Seven separate experiments, of which Fig. 3 and 4 are examples, did not give such a molecular ratio; in all cases, L and H chains increased in the 1:1 molar ratio at which these chains exist in endogenously nicked toxin.

The conclusion does not rule out the possibility of minor differences. Trypsin could have cleaved also a bond(s) close to C- and/or N-terminals. Such an event would go unrecognized if PAGE cannot detect the very small fragment(s) and cannot distinguish the large piece(s) from the endogenously generated H or L (or both) chain because of similarities in size.

Aside from lower activations of type E and B toxins (3, 7) by TLE than by trypsin, the molecular changes induced by the enzymes are strikingly different. Trypsin cleaved unnicked type E toxin into H and L chains, but a molecular change accompanying the toxicity increase from TLE action could not be detected. Thus, the bonds attacked by the two enzymes must be different in spite of the closely similar substrate specificities of these enzymes (6).

Treating the purified type B toxin samples with TLE did not give a detectable toxicity increase or evidence of conversion of unnicked molecules to the nicked form (unpublished data). Since these toxin molecules were developed in culture with TLE activity (3), unnicked type E toxin was taken as a model substrate that was not previously exposed to TLE (7). If action of TLE on type E toxin is representative of its effect on toxins of proteolytic *C. botulinum* cultures, the presence of only nicked, completely activated toxin forms in type A and F cultures that have been incubated for maximum toxicity must occur because these cultures have another enzyme(s) capable of carrying out the nicking to give the H and L chains. This inference, that complete natural activation requires the action of at least two enzymes, is consistent with our previous hypothesis (7).

Since the partially purified type B toxin preparation obtained from a 24-h culture, unlike those purified from a 96- or 168-h culture, was activated by TLE (3), it is assumed at present that the nicked and unnicked toxins in purified type B preparations have been endogenously acted upon by TLE.

We can offer only speculation as to why unnicked toxin is present in a proteolytic culture that has been incubated for as long as 7 days. The presence of nicked toxin shows that the culture has the mechanism for nicking native toxin, but complete conversion does not occur. One possibility would be that two kinds of toxic molecules are synthesized; only one is susceptible to endogenous "nicking enzyme," but both are cleaved by trypsin. Alternatively, the relatively high concentration of an inhibitor of trypsin that accumulates in type B cultures (9) might prevent nicking of toxin that is elaborated during the late stages of culture growth.

ACKNOWLEDGMENTS

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