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Activation of Botulinum Toxins in the Absence of Nicking

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The derivative toxins purified from cultures of proteolytic strains of *Clostridium botulinum* types A and F were found to have been only partially nicked but were fully activated. Trypsinization of *C. botulinum* type B derivative toxin at pH 6.0 resulted in simultaneous activation and nicking, whereas at pH 4.5, activation preceded nicking. The toxin was split by trypsin at pH 6.0 into two fragments with molecular weights of 112,000 and 57,000. The toxin contained at least three trypsin-sensitive peptide bonds, one of which was more sensitive than the others at pH 6.0. These results indicate that activation of botulinum toxins by trypsin or endogenous protease(s) is not a direct result of nicking.

It has been suggested that activation of botulinum toxin entails cleavage of a certain peptide bond or nicking in the single polypeptide chain of the toxic component. This nicking can be demonstrated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of trypsinized type E derivative toxin or of endogenously activated type A and B derivative toxins under reducing conditions (4, 14). The nicked derivative toxin consists of two subunits with approximate molecular weights of 100,000 and 50,000 linked together with a disulfide bridge(s).

We preliminarily observed in SDS-gel electrophoresis that the molecules of the derivative toxin of proteolytic *C. botulinum* type F strain Langeland were being only partially nicked and that trypsinization of such toxin caused no increase in toxicity. A similar discrepancy was mentioned by DasGupta and Sugiyama (5). Although activation of botulinum toxin was ascribed to the nicking (4), the discrepancy observed with type F toxin raised an important question as to whether the nicking in the toxin molecule by trypsin or endogenous protease(s) is really the basis for the increase in the toxicity. The studies presented here were performed to answer this question.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. For purification of endogenously activated type A, B, and F progenitor toxins, *C. botulinum* type A strain Hall, type B strain Okra, and type F strain Langeland were grown in the toxin production medium (10) at 30°C for 3, 4, and 4 days, respectively. To obtain type B progenitor toxin that can be activated by trypsinization, type B strain Okra was grown in the same medium for 40 h at 30°C.

Purification of type A, B, E, and F progenitor toxin. Progenitor toxins of *C. botulinum* types A, B, and F were purified by essentially the same method as that described for type F (10), with the following modification; at step 2, 0.1 M acetate buffer, pH 6.0, containing 0.5 M NaCl was used to extract the toxin from the acid precipitate, and at step 3, an equal volume of 0.1 M citrate buffer, pH 4.5, containing 0.5 M NaCl was added to the toxic extract before the addition of protamine. At the final step of purification, type A and B toxins were each eluted in two toxic peaks from a column of Sephadex G-200 as reported before (9, 13). The second peak, termed M (medium-sized) toxin for both types A and B, was used for obtaining derivative toxin.

Type E progenitor toxin was purified from the bacterial cells of a strain of German sprats (7) by the method of Kitamura et al. (8).

Separation of type A, B, E, and F derivative toxins. The derivative toxins of types A, B, E, and F were separated by the method described for type F (11).

SDS-gel electrophoresis. SDS-gel electrophoresis and molecular weight determination were performed in 5% polyacrylamide gel according to Dunker and Rueckert (6). Polyacrylamide gel was prepared by mixing 4.7 volumes of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS (PB-SDS) with 1.0 volume of a 30% acrylamide monomer solution in PB-SDS containing 0.1% *N,N,N',N'*-tetramethylenediamine and 0.3 volumes of 1% ammonium persulfate in PB-SDS. The ratio of acrylamide to methylenebisacrylamide was 29:1 (wt/wt). A sample was added to an equal volume of a solution containing 2% mercaptoethanol, 8 M urea, and 2% SDS. The mixture was heated for 2 min in a boiling-water bath, and then a 50-μl portion of the sample containing 2 to 10 μg of protein was applied to a polyacrylamide gel column (0.6 by 8 cm). Electrophoresis was carried out at 6 mA per column for 180 to 210 min. After electrophoresis, the gel columns were stained with 0.1% Coomasie brilliant blue in 50% trichloroacetic acid for 1 h at 40°C and destrained with 7% acetic acid at room temperature.

Molecular weight determination by SDS-gel electrophoresis. To determine the molecular weight

from the mobility rate relative to that of chymotrypsinogen A (molecular weight = 25,000) in SDS-gel electrophoresis, the following protein standards were used; β-galactosidase (molecular weight = 130,000), phosphorylase a (molecular weight = 94,000), bovine serum albumin (molecular weight = 66,000), and ovalbumin (molecular weight = 45,000).

Activation and toxicity assay. Trypsinization was carried out in 0.1 M acetate buffer, pH 6.0, for 30 min at 35°C, unless indicated otherwise. Whole cultures of types A, B, E, and F were treated with trypsin at a final concentration of 0.2 mg/ml. Purified progenitor and derivative toxins were activated at toxin-to-trypsin ratios of 5 to 1 and 20 to 1, respectively.

Toxicity was assayed by the time-to-death method by intravenous injection into mice (2, 12).

The trypsin concentration was estimated from the extinction coefficient ($E^{1\%}_{280\ nm}$) of 15.6 (3).

Chemicals. Phosphorylase a and ovalbumin (Pentex Inc., Kankakee, Ill.), β-galactosidase (Boehringer Mannheim GmbH, Mannheim, West Germany), bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago, Ill.), and chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.) were used as protein standards for determination of the molecular weight by SDS-gel electrophoresis. Soybean trypsin inhibitor was the product of Worthington Biochemical Co., Freehold, N.J.; trypsin (type III, twice crystallized) was from Sigma Chemical Co., and SDS was from Wako Pure Chemical Industries, Osaka.

TABLE 1. *Activation of different preparations of type A, B, E, and F toxins*

Toxin	LD_{50} ($\times 10^{-7}$) per 100 ml of culture fluid or LD_{50} ($\times 10^{-8}$) per mg of N (purified material)		Activation ratio
	Untreated	Typsinized	
Type A (Hall)			
Culture[a]	34	33	0.97
Progenitor	4.2	3.9	0.93
Derivative	9.0	8.5	0.94
Type F (Langeland)			
Culture[b]	2.2	2.3	1.05
Progenitor	1.2	1.1	0.92
Derivative	2.4	1.6	0.67
Type B (Okra)			
Culture[b]	5.0	55	11.0
Progenitor	1.5	6.2	4.25
Derivative	3.3	9.4	2.83
Type E (German sprats)			
Culture[b]	0.0018	0.51	283
Progenitor	0.0015	0.55	367
Derivative	0.028	1.56	54

[a] Incubated for 73 h.
[b] Incubated for 97 h.

RESULTS

State of nicking in unactivated and endogenously activated toxins. Toxicities of type A, B, E, and F whole cultures and purified progenitor and derivative toxins before and after trypsinization are shown in Table 1. The toxins derived from proteolytic type B and non-proteolytic type E strains were potentiated upon trypsinization, whereas those from proteolytic type A and F strains were not.

The patterns of SDS-gel electrophoreses of type A and F derivative toxins, having been fully activated endogenously during incubation of the cultures, showed only partial nicking of the toxin molecules (Fig. 1). The molecular weights of the derivative toxins and their dissociated fragments were 153,000, 99,000, and 51,000 for type A and 157,000, 108,000, and 51,000 for type F, respectively.

By SDS-gel electrophoreses under the same conditions, type B derivative toxin showed only partial dissociation into fragments, and type E derivative toxin showed no dissociation (Fig. 2).

Dissociation of activation and nicking in type B derivative toxin. (i) Effect of pH. Type B derivative toxin was trypsinized at different pH values for 20 min at 35°C; each product was applied to an SDS-polyacrylamide gel column. The optimum pH for activation of type B derivative toxin was 6.0 (Fig. 3), where complete nicking occurred. The molecular weights of type B derivative toxin and its dissociated fragments were 163,000, 112,000, and 57,000, respectively. Activation of type B derivative toxin was also attainable at pH 4.5, being accompanied by only partial nicking of the toxin molecules (Fig. 4). At pH 7.0 and 8.0, where trypsin acts most strongly, type B derivative toxin was cleaved into at least three fragments. Two of these fragments migrated to the same relative positions as did the two fragments produced at pH 6.0; the third one, with a molecular weight of approximately 142,000, emerged only when trypsinization was performed at pH values above 7.

(ii) Effect of trypsin concentration. Maximum activation was attained by incubation for 20 min at pH 6.0 at the trypsin-to-toxin ratio of 1:10 (Fig. 5). Trypsinization under these conditions or at higher trypsin concentrations, however, caused a decrease in the color density of the band of the larger fragment (molecular weight, 112,000). The trypsin-to-toxin ratio of 1:20 resulted in the same relative color densities of the two fragments (Fig. 6, gel column). These results indicate that the higher-molecular-weight fragment is more susceptible to

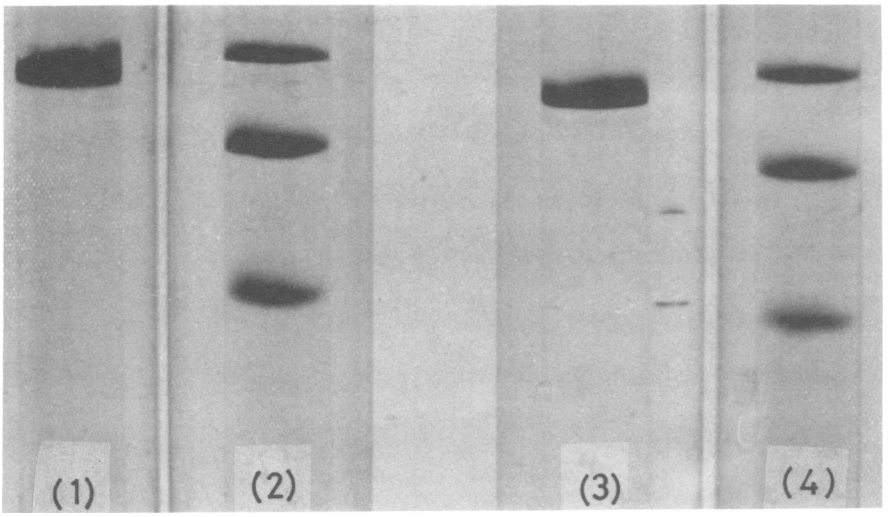

FIG. 1. *Electrophoretic patterns of type A and F derivative toxins. Type A and F derivative toxins before and after reduction were subjected to SDS-polyacrylamide gel electrophoresis. (1) Type A toxin before reduction, (2) type A toxin after reduction, (3) type F toxin before reduction, and (4) type F toxin after reduction.*

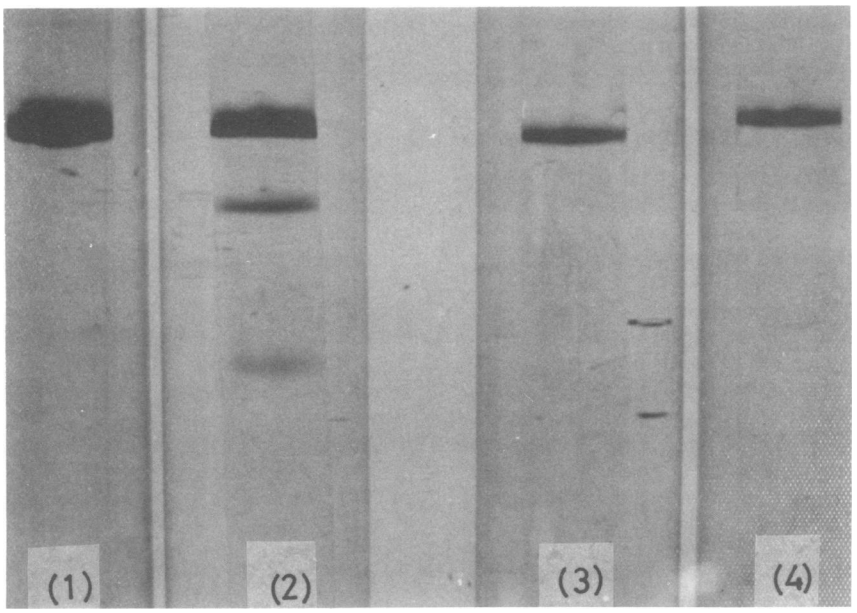

FIG. 2. *Electrophoretic patterns of type B and E derivative toxins. Type B and E derivative toxins before and after reduction were subjected to SDS-polyacrylamide gel electrophoresis. (1) Type B toxin before reduction, (2) type B toxin after reduction, (3) type E toxin before reduction, and (4) type E toxin after reduction.*

trypsin than the lower-molecular-weight fragment.

(iii) Time courses of activation and nicking at pH 4.5 and 6.0. The relationship between activation and nicking was further scrutinized by performing trypsinization on the type B derivative toxin at pH 6.0 and 4.5. At pH 4.5, activation proceeded more slowly than at pH 6.0, but after 60 min of incubation, the toxicity reached the same level as the maximum toxicity

attained in 20 min at pH 6.0 (Fig. 7). The SDS-gel electrophoretic patterns show that under these conditions, the nicking in type B derivative toxin proceeded faster at pH 6.0 (Fig. 8a) than at pH 4.5 (Fig. 8b) and that a certain section of each toxin molecule remained unnicked at pH 4.5, although the toxicity reached the same level as the maximum toxicity attained at pH 6.0. On the other hand, all the molecules of the derivative toxin were nicked within 20 min at pH 6.0, when full activation was attained.

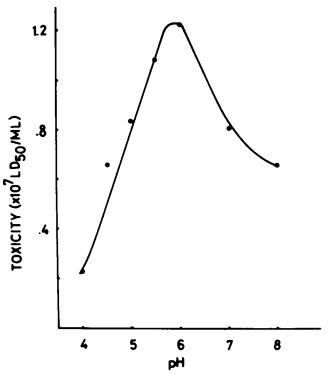

FIG. 3. *Activation of type B derivative toxin by trypsin at different pH values. Type B derivative toxin (20 μg in 100 μl of the reaction mixture) was treated with trypsin (1 μg in 100 μl of the reaction mixture) for 20 min at 35°C. The enzyme action was terminated by adding 10 μl of 0.02% soybean trypsin inhibitor to 100 μl of the reaction mixture. Two 30-μl portions were removed from each mixture. One of them was diluted with 0.1 M acetate buffer, pH 6.0, and injected into mice to determine the toxicity. The other was subjected to SDS-gel electrophoresis (Fig. 4). The buffers used were: 0.1 M acetate buffer, pH 4.0, 4.5, 5.0, 5.5, and 6.0; 0.05 M phosphate buffer, pH 7.0 and 8.0.*

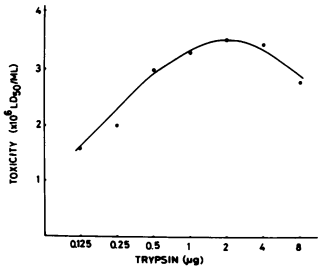

FIG. 5. *Activation of type B derivative toxin (20 μg in 100 μl of the reaction mixture) by trypsin at different trypsin concentrations. The trypsin action was terminated by adding soybean trypsin inhibitor in twice the quantity of trypsin. Two 30-μl portions were removed from each mixture. One of them was diluted with 0.1 M acetate buffer, pH 6.0, and injected into mice to determine the toxicity. The other was subjected to SDS-gel electrophoresis (Fig. 6).*

DISCUSSION

The nicking in botulinum toxin was first reported by Beers and Reich (1) with type B derivative toxin purified from proteolytic strain Okra. According to them, type B toxin with a

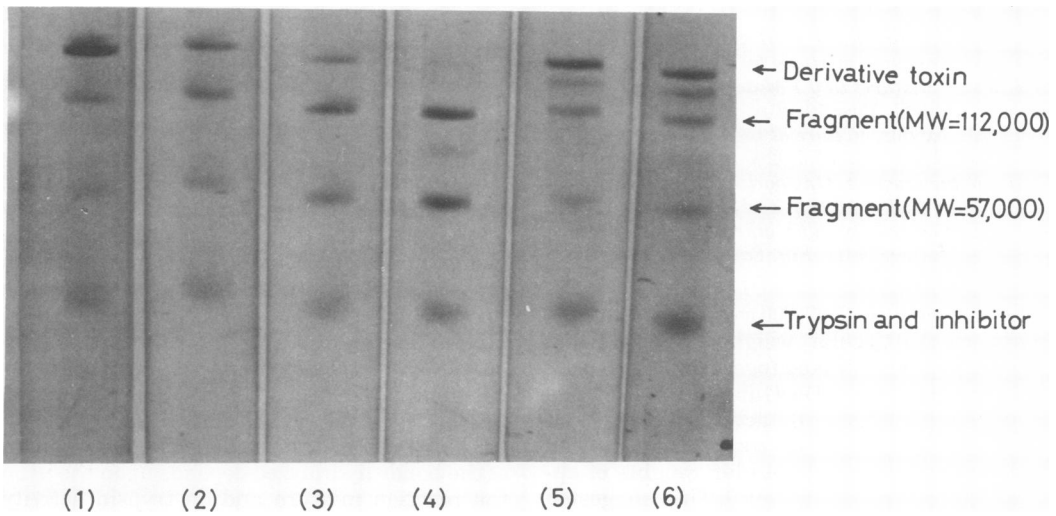

FIG. 4. *Electrophoretic patterns of type B derivative toxin treated with trypsin at different pH values. Type B derivative toxin was trypsinized in 0.1 M acetate buffer, pH 4.5 (1), 5.0 (2), 5.5 (3), and 6.0 (4), and in 0.05 M phosphate buffer, pH 7.0 (5) and 8.0 (6). MW, Molecular weight. Other details are given in the legend to Fig. 3.*

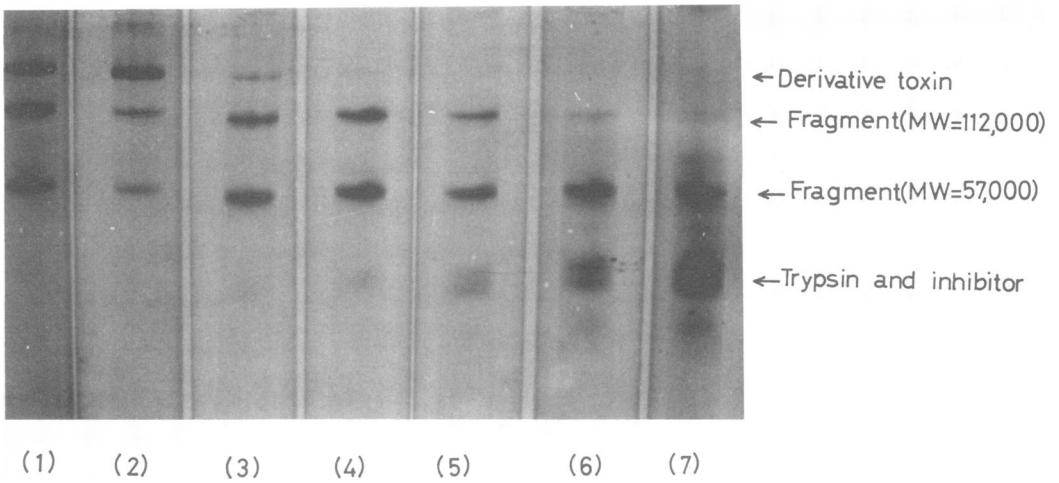

FIG. 6. *Electrophoretic patterns of type B derivative toxin treated for 20 min at 35°C with trypsin at different concentrations in 0.1 M acetate buffer, pH 6.0. The trypsin contents of 100 μl of the reaction mixture containing 20 μg of the toxin were: 0.125 μg (1), 0.25 μg (2), 0.5 μg (3), 1.0 μg (4), 2.0 μg (5), 4.0 μg (6), and 8.0 μg (7). MW, Molecular weight. Other details are given in the legend to Fig. 5.*

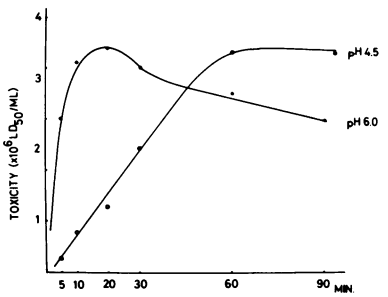

FIG. 7. *Activation of type B derivative toxin (200 μg/ml of the reaction mixture) by trypsin (10 μg/ml of the reaction mixture) in 0.1 M acetate buffer, pH 6.0 and 4.5, at 35°C. Two 50-μl portions were removed from each reaction mixture at the indicated times; to each was added 5 μl of 0.02% trypsin inhibitor to terminate the reaction. One portion was diluted with 0.1 M acetate buffer, pH 6.0, and injected into mice to determine the toxicity. The other was subjected to SDS-gel electrophoresis (Fig. 8a and b).*

molecular weight of 167,000 consists of two fragments with molecular weights of 59,000 and 104,000, which are linked together with at least one disulfide bridge. DasGupta and Sugiyama (4) showed that naturally activated or trypsinized botulinum toxins are commonly composed of two subunits with molecular weights of approximately 100,000 and 50,000. They suggested also that these common subunits are formed by activation with either trypsin or an endogenous trypsin-like enzyme. More recently, the same authors (5) described activation of type E toxin in the absence of nicking by a trypsin-like enzyme produced by the same proteolytic type B strain.

Trypsinization of derivative toxin at different pH values showed that full activation occurs at pH 4.5, being accompanied by only partial nicking. Taking advantage of such dissociations, we attempted to differentiate activation from nicking. At pH 6.0, the increase in the toxicity seemed to parallel the nicking, whereas at pH 4.5, the nicking was incomplete but the increment in toxicity was complete, though it took a longer time to attain the maximum toxicity than at pH 6.0.

By trypsin digestion, type B derivative toxin is first split into two fragments with molecular weights of 112,000 and 57,000. As the trypsin concentration or the incubation time was increased, the 112,000-molecular-weight fragment was digested further into a smaller peptide(s), when the toxin molecule possessed the maximum toxicity (unless it was fragmented by reduction). At slight alkalinity, near the optimum pH for trypsin, the third fragment, with a molecular weight of 142,000, emerged. The appearance of the third fragment and of a smaller fragment(s) indicates that at least three peptide bonds in the type B derivative toxin chain are susceptible to trypsin and that the number of the bonds hydrolyzed depends upon the pH of the reaction mixture and on trypsin activity. The fact that complete nicking into the two fragments resulted at pH 6.0 with a low trypsin concentration suggests that the particular single peptide bond is more susceptible to trypsin than the others. Partial nicking was demon-

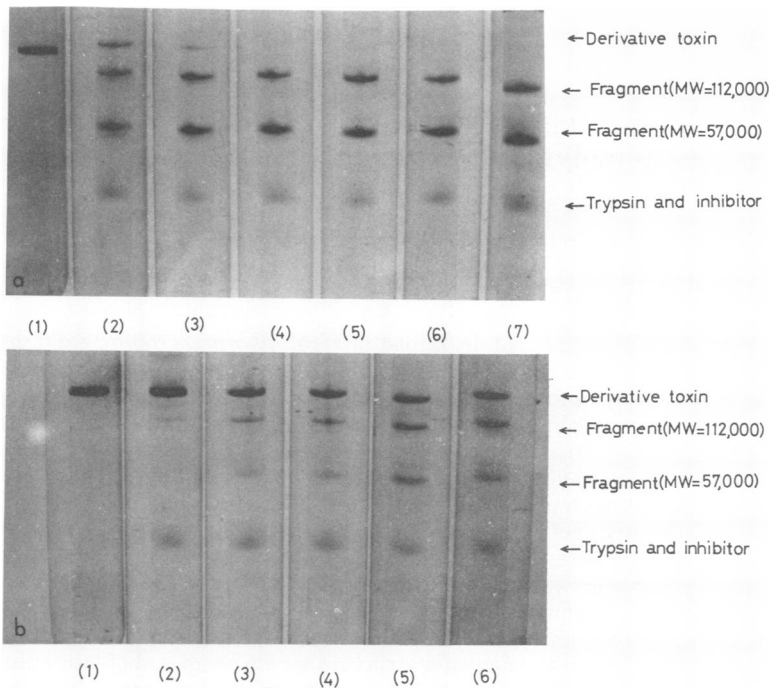

FIG. 8. *Electrophoretic patterns of type B derivative toxin trypsinized in 0.1 M acetate buffer, pH 6.0 (a) or pH 4.5 (b), for different periods at 35°C. Samples were removed and subjected to SDS-gel electrophoresis after (a), (1) 0 min, (2) 5 min, (3) 10 min, (4) 20 min, (5) 30 min, (6) 60 min, and (7) 90 min; (b), (1) 0 min, (2) 5 min, (3) 10 min, (4) 30 min, (5) 60 min, and (6) 90 min. MW, Molecular weight. Other details are given in Fig. 7.*

strated with fully activated type A and F derivative toxins elaborated by proteolytic strains producing the enzyme activating the toxin. All these facts strongly indicate that nicking occurs only incidentally and is not directly associated with activation.

Partial nicking demonstrated in toxin fully activated by either trypsin or the endogenous protease suggests that activation precedes the nicking in the toxin molecule.

ACKNOWLEDGMENT

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