ACTIVATION PHENOMENON OF CLOSTRIDIUM BOTULINUM TYPE E TOXIN

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Abstract

Gerwing, Julia (The University of British Columbia, Vancouver, Canada), Claude E. Dolman, and David A. Arnott. Activation phenomenon of Clostridium botulinum type E toxin. J. Bacteriol. 84:302-306. 1962—Highly purified preparations of both nonactivated and trypsin-activated type E botulinus toxins have been analyzed in an ultracentrifuge. Untreated botulinus type E toxin was found to have a sedimentation constant (S20,w) of 5.6 Svedberg units, whereas trypsin-activated toxins would not form a boundary under identical conditions. These and other considerations indicate that the mechanism of trypsic activation involves a fragmentation process whereby more toxic sites become exposed.

The data to be presented here support the first of these alternatives, for studies of the sedimentation rates of both trypsin-activated and nonactivated type E toxins indicate that the activated toxin molecules are considerably smaller than those of the original toxin.

Materials and Methods

The strains, materials, and methods used in this work were as previously described (Gerwing, Dolman, and Arnott, 1961). Ultracentrifugation

FIG. 1. Second elution of type E toxin through DEAE cellulose. Material used represents nine pooled preparations of toxin purified by previous elution. Broken line: 280-μm absorption; solid line: 260-μm absorption.
was carried out in a Beckman/Spinco model E analytical centrifuge.

RESULTS

We have shown previously that highly purified preparations of both nonactivated and trypsin-activated type E toxins can be produced by ethanol precipitation of the toxins followed by elution through diethylaminoethyl (DEAE) cellulose columns (Gerwing et al., 1961). In the experiments reported here, to obtain a further degree of purification, the toxic peak samples obtained from nine primary fractionations were pooled, dialyzed against running water for 24 hr, and dried by lyophilization. The dried preparations were resuspended in distilled water and eluted through DEAE cellulose with sodium acetate buffer (pH 6.5) in ascending concentration.

The results of this procedure, carried out on nonactivated and trypsin-activated toxins, are summarized in Fig. 1 and 2, respectively. In each case, the further purification permitted the separation of another component, which was not toxic. [The potencies of the toxic fractions were only slightly higher than those reported previously by us (Gerwing et al., 1961), i.e., approximately $6 \times 10^4$ and $1 \times 10^7$ MLD per mg of N$_2$ for nonactivated and activated toxin, respectively. This relatively small improvement in potency, despite the elimination of a nontoxic component, can be explained in terms of the demonstrable loss of toxicity suffered by these highly purified preparations in the course of lyophilization.] Once again, the toxic peak samples were dialyzed against running water for 24 hr and dried by lyophilization. These preparations were resuspended in physiological saline at a concentration of 2% protein and used for ultracentrifuge analysis.

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**FIG. 2.** Second elution of trypsin-activated type E toxin through DEAE cellulose. Material used represents nine pooled preparations of toxin purified by previous elution. Broken line: 280-μ absorption; solid line: 222 μm absorption.

**FIG. 3.** Schlieren diagram showing ultracentrifuge behavior of type E toxin. Pictures taken 8 min apart at 70° angle. Material represents 1.78% protein. Speed of centrifugation: 259,700 × g; temperature: 12 C.
The Schlieren diagrams of the ultracentrifuge behavior of the nonactivated toxin are shown in Fig. 3. This preparation contained two components, with sedimentation constants (S\textsubscript{20,w}) of 5.6 and 1.1 Svedberg units. To establish whether both of these components were toxic, a second ultracentrifuge run was made using a moving partition separation centerpiece. This run was made at 260,000 \times g for 90 min. Separation of the two components was thus effected, and the results showed that only the heavier component (S\textsubscript{20,w} = 5.6) contained toxic activity. Trypsin-activated toxin was similarly analyzed, and gave Schlieren diagrams (Fig. 4) which suggested that the activated material was too small to form a moving boundary under the conditions of the experiment.

**DISCUSSION**

The ultracentrifuge analyses carried out on purified preparations of nonactivated and trypsin-activated botulinus type E toxins appear to throw some light on the tryptic-activation mechanism. In our hands, nonactivated toxin showed a sedimentation constant within the range of many proteins and formed a moving boundary easily discernible with the Schlieren optical system. Under the same conditions, activated toxins displayed no boundary, but seemed to form a concentration gradient at the meniscus of the centerpiece. The trypsin-activated toxin prepared by Fiock, Yarinsky, and Duff (1961) showed the presence of two components in the ultracentrifuge, with sedimentation constants of 12.5 and 4.7 Svedberg units. As these authors did not allude to any final verification of toxicity, it seems justifiable to suggest that at least one of their components might have proved nontoxic. In any case, their preparations were not comparable to ours, since theirs were derived from whole cultures and subjected to relatively crude purification procedures.

Our data suggest that type E botulinus toxin, in the course of activation by trypsin, is broken into smaller components, probably not of uniform size, which are too small to form a boundary in the ultracentrifuge under the stated conditions. (This suggestion is supported by our recent observation that toxic molecules will dialyze into distilled water from trypsin-activated toxins, whereas untreated toxins are not dialyzable). In other words, the process of trypsic activation probably entails some degree of molecular fragmentation, with consequent increase in the number of active groups. In some respects, the mechanism of types A and B toxin production may be analogous. According to Bonventre and Kempe (1960), in the course of cytolysis, endogenous proteolytic enzymes are released along with large protoxin molecules, and may cause partial degradation of the latter with consequent exposure of more toxic sites.

Two deductions from the above concept seem warranted. First, bacterial or nonbacterial enzymes may carry molecular degradation of botulinus toxin (regardless of type) to a stage where toxicity declines and eventually disappears, thus accounting, in part at least, for the fickle stability of these toxins. Second, a preliminary enzyme activation of certain types of toxins might be extended by exposing the products, under appropriate conditions, to another proteolytic enzyme. A few experimental observations bearing on this latter possibility can be cited.

Sakaguchi and Tohyama (1955a) isolated from botulogenic fish a feebly toxigenic type E strain of *C. botulinum*, and also a nontoxic, proteolytic organism of the *Clostridium* genus, the latter being regarded by them as a contaminant. When
both cultures were grown together, toxin production was increased about 100-fold; but when the "contaminant" was grown in mixed culture with a type A strain of *C. botulinum*, the latter's toxicogenic capacity was unaltered. The same authors (Sakaguchi and Tohyama, 1955b) later ascribed the enhancement of type E toxigenicity to a filterable proteinase-like substance, elaborated by the "contaminant" culture, and acting optimally at pH 5.3. In our view, this "contaminant" might have been a proteolytic ("TP") mutant of its nonproteolytic type E fellow-isolate (Dolman, 1957).

Apparently, trypsin is more effective than the foregoing bacterial enzymes as a secondary activator of botulinus toxins. We have shown, for example, that the variable degree of activation undergone by type E toxin on exposure to the proteolytic "TP" mutant may be further enhanced by incubation with 0.1% trypsin for 1 hr at 37 C. Again, trypsic activation ratios ranging from about 10:1 to 30:1 were obtained with different preparations of type B toxin, irrespective of the proteolytic or nonproteolytic character (Dolman et al., 1960) of their parent cultures.

The molecular fragmentation hypothesis and its corollaries, as applied to type E toxin-activation processes, require further experimental verification and elucidation. In particular, a careful study of the effects of activation upon the antitoxin-binding power of the toxin might prove very revealing. Meanwhile, the results of centrifugal analysis reported above seem difficult to explain on any other basis. An alternative suggestion made in our previous paper (Gerwing et al., 1961), that trypsin acts here by cleavage of specific amino-acid bonds, with consequent molecular "unfolding," seems incompatible with findings which point to a substantial reduction in size of the activated toxin molecule. The "potentiation" hypothesis, i.e., that trypsin does not attack the toxin molecule directly but merely acts upon some other constituent in toxic filtrates to yield a potentiating factor, also can be ruled out on the ground that it entails no reduction in size of the toxin molecule. Moreover, as pointed out by Duff et al. (1956), type E toxin can still be activated with trypsin after it has been considerably purified and hence presumably freed from possible potentiators.

Finally, Sakaguchi and Sakaguchi (1959) have postulated a masking group, detachable by trypsin from the type E protoxin or precursor molecule. According to them, in the activation process the toxin moiety of the precursor molecule remains intact; but a specific kind of molecular fragmentation occurs involving the separation of a group containing ribonucleic acid, and also a masking group. They ascribed further masking properties to the nucleic acid component itself. The validity of this hypothesis can be challenged on two grounds. The detachment of specific inhibitory fragments from a toxin precursor whose molecules appear to be homogeneous (Fig. 3) should yield activated toxin of reduced but still fairly even molecular size, an expectation which seems incompatible with our findings (Fig. 4). An even more serious objection to the above conception, as pointed out previously (Gerwing et al., 1961), is our inability to detect nucleic acids in partially purified preparations, either before or after trypsic activation. We believe the discrepancies may be due to our preparations being derived from culture filtrates relatively free from bacterial cytoplasm, whereas the Sakaguchis used culture extracts derived by centrifugation, presumably containing substantial amounts of ribonucleic acid of cell-wall origin.

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LITERATURE CITED


