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In Vitro Acetylcholinesterase Inhibition by Type A Botulinum Toxin

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Type A botulinum toxin was studied for its ability to inhibit the action of acetylcholinesterase. The chromogenic substrate, indophenyl acetate, was used for assay of enzyme activity. Inhibition of enzyme function was detected through use of both 6.6×10^{-6} mg (20 LD_{50}) and 6.6×10^{-10} mg (2×10^{-3} LD_{50}) of type A botulinal toxin. Control assays were performed by use of both homologous antitoxin and heterologous antitoxins (types B and E). Enzyme inhibition was effectively prevented by use of homologous antitoxin only. The inhibition noted was specific and reproducible for given substrate, enzyme, and toxin concentrations.

Various techniques have been investigated for detection of botulinal toxin in foods, biological materials, and other vehicles in search for a specific, rapid, and sensitive test for each of the five common serotypes of this toxin. Early work has been summarized in a recent review on botulism (8). The best of the early methods was the mouse toxicity test, for which specific antitoxin was used to establish the toxin serotype by immune-protection control tests, but this procedure was slow, despite its sensitivity and specificity. In more recent research, the discovery of hemagglutination of certain kinds of erythrocytes by toxin-containing preparations (6) seemed to interested workers in this area to be a possible approach until Lamanna and Lowenthal (7) demonstrated that hemagglutinin activity and botulinal toxin activity were not associated in a single molecule. Boroff (1, 2) made the interesting observation that native botulinal toxins showed specific fluorescence in ultraviolet light, but it was later demonstrated conclusively by Schantz, Stefanye, and Spero (9) that toxicity was lost in the toxin preparations that still retained fluorescence during chemical treatment. Vermelia (*unpublished data*) demonstrated the specificity of a gel immunoprecipitin test for the botulinal toxins, but this test required relatively large amounts of toxin for clear results, and also required an extended incubation period for the positive test to develop.

Well-documented reports have shown that botulinal toxin acts on the cholinergic synapses of the motor nerve terminals through an irreversible inactivation of the presynaptic release mechanism of acetylcholine (3). However, the mechanics of the inhibition have not been determined as yet. Since acetylcholinesterase plays a very important role in the transmission of stimuli, and since this transmission is blocked by the toxin, it seemed possible that the toxin has a direct effect on acetylcholinesterase itself. However, there is no in vivo evidence that the toxin interacts with acetylcholinesterase. Chromogenic substrates such as paroxon and similar insecticides have been developed for the detection of inhibitors of this enzyme (5). In the present report, botulinum toxin has been tested as an inhibitor of acetylcholinesterase action on the chromogenic substrate indophenyl acetate.

MATERIALS AND METHODS

Reagents. The acetylcholinesterase (type III, electric eel, lot 1158-8551) used in this study was purchased from Sigma Chemical Co., St. Louis, Mo. Indophenyl acetate (IPA) was purchased from Mann Research Laboratories, Inc., New York, N.Y. Crystalline type A toxin was kindly supplied by Edward J. Schantz, U.S. Army Biological Laboratories, Fort Detrick, Md. Purified crystalline antitoxin, types A, B, and E, was supplied by the Communicable Disease Center, Atlanta, Ga. Bovine plasma albumin was supplied by Manuel Coria, National Animal Disease Laboratories, Ames, Iowa.

Mouse LD_{50} assay of type A botulinal toxin. The crystalline toxin was reconstituted and diluted for use in 0.9% NaCl. Ten albino mice, weighing 20 to 22 g each, were injected intraperitoneally with 0.1 ml of each dilution of botulinal toxin. Number of deaths was recorded at the end of 4 days, and LD_{50} values were determined by the method of Weiss (10) after three replications of the entire assay had been performed.

In vitro acetylcholinesterase assay. The acetylcholinesterase stock solution was prepared by diluting the purified enzyme preparation in Clark and Lubs buffer (4) at *p*H 8.0, such that 1 ml of the stock

solution contained 50 units of enzyme. One unit of enzyme activity is defined by Sigma Chemical Co. as that quantity of enzyme capable of causing hydrolysis of 10.1 μg of acetylcholine per min at 37 C. For stabilization of the enzyme, bovine plasma albumin was added to give a final concentration of 0.4% (v/v). For the assay, an appropriate amount of purified IPA was dissolved in 95% ethyl alcohol and was then diluted in ethyl alcohol to 25 ml in a volumetric flask. Samples of this stock solution were further diluted to give a final substrate concentration of 1.3×10^{-6} M.

The method of Kramer and Gamson (5) was used for assay of acetylcholinesterase activity. All assays were run on a Gilford model 2000 recording spectrophotometer by use of cuvettes with a 1-cm light path, and light of 625 mμ wavelength. The enzyme substrate reaction mixture consisted of 1.3×10^{-6} M IPA, acetylcholinesterase in concentrations of 0.5 and 5.0 μ/ml, and type A botulinal toxin at concentrations of 6.6×10^{-6} mg/ml (20 LD_{50}) and 6.6×10^{-10} mg/ml (2×10^{-3} LD_{50}). For blocking of toxicity of toxin by specific antitoxin in control tests, type A antitoxin was diluted to titer, i.e., to the point at which it would just neutralize the quantity of toxin used in the assays. Types B and E antitoxin were tested in parallel with type A antitoxin with due consideration given to the lower control activities of types B and E antitoxin, to detect any possible cross-reaction with the botulinum A toxin. The enzyme, substrate, toxin, and antitoxin, in combinations indicated in Table 1, were used as reaction mixtures and were placed in spectrophotometer cuvettes which were maintained at 25 C during optical measurements. Optical density (OD) changes were automatically recorded against time.

RESULTS AND DISCUSSION

The botulinal A toxin had a potency of 3×10^{10} mouse LD_{50}/g. which agreed with the prelyophilization value found by Schantz (*personal communication*). The variability over three replications was very small, indicating that maximal precision was obtained in determining the estimate of toxin potency.

TABLE 1. *Final optical density values at 625 mμ obtained at specified time intervals by use of indophenyl acetate (IPA), acetylcholinesterase (Ac'ase), types A, B, and E antitoxin, and type A botulinum toxin in enzyme inhibition studies*

Reaction mixture components and concentrations[a]	OD	Time (min)	Difference OD units[b]
Ac'ase[c] + IPA + 3.3×10^{-6} mg of toxin	.409	48	—
Ac'ase + IPA	.560	48	.151
Ac'ase + IPA + 3.3×10^{-6} mg of toxin + A antitoxin[d]	.564	48	—
Ac'ase + IPA	.560	48	.004
Ac'ase + IPA + 3.3×10^{-6} mg of toxin + A antitoxin[e]	.517	48	—
Ac'ase + IPA	.560	48	.043
Ac'ase + IPA + 3.3×10^{-6} mg of toxin + B antitoxin	.441	48	—
Ac'ase + IPA	.560	48	.119
Ac'ase + IPA + 3.3×10^{-6} mg of toxin + E antitoxin	.426	48	—
Ac'ase + IPA	.560	48	.134
Ac'ase + IPA + 3.3×10^{-10} mg of toxin	.457	48	—
Ac'ase + IPA	.560	48	.103
Ac'ase (0.5 μ/ml) + IPA + 3.3×10^{-10} mg of toxin	.382	120	—
Ac'ase (0.5 μ/ml) + IPA	.560	120	.178
Ac'ase + IPA + 3.3×10^{-10} mg of toxin + A antitoxin	.551	48	—
Ac'ase + IPA	.560	48	.009
Ac'ase + IPA + 3.3×10^{-10} mg of toxin + B antitoxin	.460	48	—
Ac'ase + IPA	.560	48	.100
Ac'ase + IPA + 3.3×10^{-10} mg of toxin + E antitoxin	.458	48	—
Ac'ase + IPA	.560	48	.102

[a] Final volume, 3.0 ml per cuvette.

[b] Difference in the OD of the cuvette without toxin and the cuvette with toxin or with toxin and antitoxin, as specified.

[c] Concentration of Ac'ase was 5.0 μg/ml unless otherwise specified; toxin and enzyme were incubated for 10 min prior to IPA addition except where noted.

[d] Toxin and antitoxin were incubated for 10 min prior to addition of enzyme except where noted.

[e] Toxin and Ac'ase were incubated for 30 min at 25 C before being added to the reaction mixture.

There was a definite and reproducible inhibition of acetylcholinesterase activity by the botulinal toxin appearing as a difference in OD values in test versus control readings over the time interval specified (Table 1). Indeed, the standard slopes of the dose-response curves, obtained directly, were significantly different. The inhibition by the toxin was presumably due to a binding with, or a masking of, either or both the active combining site and the specificity site on the enzyme molecule. The addition of homologous antitoxin completely reversed this action and prevented all enzyme inhibition at both toxin concentrations used. Absolute specificity of the enzyme inhibition by the toxin was revealed through the ineffectiveness in blocking toxin activity by heterologous antitoxins at either toxin concentration and through the reversal of inhibition by addition of homologous antitoxin. In fact, the serological cross-reaction observed by Lamanna and Lowenthal (7) between type A toxin and type B antitoxin obviously did not occur in this system. Although acetylcholinesterase inhibition is reproducible and constant for given toxin, enzyme, and substrate concentrations, enzyme inhibition is never complete. Concentration gradients of each of the compounds in the reaction mixture will be run to determine whether complete inhibition can be achieved with a sufficiently large dose of toxin and whether decreased levels of inhibition can be achieved with more dilute toxin preparations.

It was found, in this regard, that the difference in OD (amount of inhibition of the enzyme by the toxin) could be increased by increasing the toxin concentration while keeping the enzyme and substrate concentrations constant, or by decreasing the enzyme concentration and increasing the incubation time. Also, when toxin A and enzyme were incubated for 10 min prior to the addition of antitoxin A and when this mixture was then incubated for 10 min before IPA addition, a total reversal of enzyme inhibition was obtained. However, when toxin A and enzyme were incubated for 30 min prior to the addition of antitoxin A and when this mixture was then incubated for an additional 10 min before addition of IPA, an incomplete enzyme inhibition was obtained; i.e., about half the OD difference was observed, relative to that in the toxin-enzyme system incubated for only 10 min. From these data, we speculate that there may be an action of acetylcholinesterase on the toxin such that release of small toxic, but not antigenic, fragments occurs. According to this speculation, there would be partial inhibition of enzyme activity by fragments not binding to antitoxin and whose concentration would be related to the time of incubation or of exposure of toxin to the acetylcholinesterase molecule. Our data indicate that some system, perhaps not the one above, is causing a potentiation of toxicity such that the amount of an antitoxin required for neutralization is no longer sufficient to prevent enzyme inhibition. Further work will be done to characterize this system.

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LITERATURE CITED

1. BOROFF, D. A. 1959. Studies of the toxin of *Clostridium botulinum*. IV. Fluorescence of the toxin of *Clostridium botulinum* and its relation to toxicity. Intern. Arch. Allergy Appl. Immunol. **15**:74–90.
2. BOROFF, D. A., AND J. E. FITZGERALD. 1958. Fluorescence of the toxin of *Clostridium botulinum* and its relation to toxicity. Nature **181**:751–752.
3. BROOKS, V. B. 1964. The pharmacological action of botulinum toxin, p. 105–111, U.S. Public Health Serv. Publ. 999-Fp-1.
4. CLARK, W. M. 1928. Determination of hydrogen ions, p. 200. Williams & Wilkins Co., Baltimore.
5. KRAMER, D. N., AND R. M. GAMSON. 1958. Colorimetric determination of acetylcholinesterase activity. Anal. Chem. **30**:251–254.
6. LAMANNA, C. 1948. Haemagglutination by botulinal toxin. Proc. Soc. Exptl. Biol. Med. **69**:332–336.
7. LAMANNA, C., AND J. P. LOWENTHAL. 1951. The lack of identity between hemagglutinin and the toxin of type A botulinal organism. J. Bacteriol. **61**:751–752.
8. SCHANTZ, E. J. 1964. Purification and characterization of *C. botulinum* toxins, p. 91–95. U.S. Public Health Serv. Publ. 999-Fp-1.
9. SCHANTZ, E. J., D. STEFANYE, AND L. SPERO. 1960. Observations on the fluorescence and toxicity of botulinum toxin. J. Biol. Chem. **235**:3489–3491.
10. WEISS, E. S. 1948. An abridged table of probits for use in the graphic solution of the dosage-effect curve. Am. J. Public Health **38**:22–24.