ASSAY OF TYPE A BOTULINAL TOXIN USING ACETYLCHOLINESTERASE INHIBITION

by

Rosemarie Marshall

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Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

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INTRODUCTION

Concern over the health hazard inherent in the potent toxins produced by various strains of Clostridium botulinum has spurred a great deal of interest in the elucidation of procedures for detecting the presence of these toxins in foods, for quantitating the toxins, and for determining the mechanism(s) of toxin action. Unfortunately, it has not been possible to detect and quantitate the toxin in carrier materials strictly by in vitro analytical techniques. This procedure is accomplished, at the present time, on the basis of in vivo assays using the reaction of mice or guinea pigs injected with dilutions of the suspect material. While biological assays are usually considered to be the ultimate in sensitivity, the reproducibility of results and the relatively slow reaction rates are serious limitations when a person's life may depend upon the results of the assay. For these reasons, it is of extreme importance that either a revision be made of the biological assay technique which is now used for these toxins, or a new assay system be developed.

This study was undertaken in order that a rapid, sensitive, and reproducible assay method might be devised for the detection and quantitation of the A serotype of botulinal toxin. The completed assay is an in vitro method based on the inhibition of acetylcholinesterase activity on the chromogenic substrate, indophenyl acetate, by type A botulinal toxin.

LITERATURE REVIEW

The first report in the literature pertaining to the toxic products of bacteria, in this case to those of the diphtheria bacillus, was that of Rous and Yersin (1888, p. 645), who stated: "Certain bacterial species produce/contain antigenic poisonous substances of high molecular weight giving rise to more or less characteristic lesions or symptoms, including death, when injected into susceptible animals." Indeed, this generalization could as well have been made regarding those strains of Clostridium botulinum which produce an extremely toxic protein called botulinum toxin. Van Ermengem (1897) first recognized and described botulism as a disease. He also separated the botulism syndrome from those of other types of food poisoning, identified the disease bacteriologically, and proved that it resulted from ingestion of the bacterial toxin produced by the obligately anaerobic bacillus which he named Bacillus botulinum, synonymous with the modern name, Clostridium botulinum.

Six strains of C. botulinum, A, B, C, D, E, and F, can produce six toxins and each toxin bears the same identifying letter as the strain which produces it. According to Lamanna (1959), one strain of the organism produces only one serotype of toxin; fortunately, mutation of strains to produce other serotypes is unknown. Van Heyningen (1950) describes these toxins as typical of those produced by Gram-positive bacteria. Prévot (1966) noted that the six strains are closely related, but differ from each other in the antigenic structure of their toxin and in some differences in their physiology. The antigenic nature of the toxins determines the host affected, i.e., types A, B, E, and F affect man, types C and D

do not. The physiological differences among these strains are mirrored in the difference in heat resistance of the spores (Foster et al., 1965). Characterization of these strains has been based most recently on a technique devised by Walker and Batty (1964) in which antitoxin labelled with fluorescein isothiocyanate is used to divide the six serotypes into three groups; types A, B, F; types C, D; and type E. The toxin is characterized further by using protection tests. Laboratory animals are injected with dilutions of the toxin from various strains and with protective doses of known serotypes of antitoxin. On the basis of the protection pattern, the serotype of the toxin can be determined

Boroff (1955) observed that young cultures of these strains, growing and metabolizing maximally, did not produce maximum amounts of toxin. Instead, the toxin concentration was highest after autolysis of most of the organisms. The addition of 'spent' culture medium to a young culture promoted autolysis. On the basis of these results, Boroff suggested that a lytic enzyme is involved in the release of toxin from the cells. However, no conclusive data are yet available concerning this possibility, even though the 'autolysis factor' was shown by Boroff to be heat sensitive.

In a study by Bonventre and Kempe (1960), it was hypothesized that proteolytic enzymes from the autolyzed organisms act upon 'protoxin', an extremely large molecule which is present within the intact cell. The action of the enzymes, then, is to increase toxicity by the fragmentation of the 'protoxin'. This hypothesis also had merit in view of the evidence presented earlier by Duff et al. (1956b) and Sakaguchi and

Sakaguchi (1959) showing that type E toxin could be activated by trypsin. Later, Gerwing et al. (1961) postulated that cleavage of specific amino acid bonds by tryptic deamination permitted 'unfolding' as well as fragmentation of the toxin molecule, thereby exposing more toxic sites. This work was substantiated by Wagman (1962). Evidence was later presented, however, that fragmentation into small molecules was not likely to be prerequisite to an activation process (Sakaguchi et al., 1964). The problem of how activation of toxin is accomplished still is not resolved.

According to Lamanna (1959), as little as 3.5×10^{-6} mg of type A toxin can be lethal to an adult human. Because such minute quantities of toxin are required for poisoning, it is important to gain information on the structure and configuration of the molecule so as to explain the extreme toxicity. To determine the molecular characteristics of the toxins, workers have developed methods designed for obtaining high titer crystalline preparations. Lamanna et al. (1946), working with the type A strain of C. botulinum, described a technique involving the use of chloroform as a step in the extraction procedure. Duff et al. (1957) simplified the procedure greatly by using alcohol precipitation techniques. Isolation of type B toxin has been described by Lamanna and Glassman (1947), and by Gerwing et al. (1966), and isolation of type E toxin has been described by Gordon et al. (1957). Type F toxin has not yet been purified. Extraction and purification procedures for types A, B, C, D, and E toxin are discussed quite comprehensively in a recent review by Lewis and Cassel, Jr. (1964).

As to the mode of action of botulinal toxins, Torda and Wolff (1946)

state that the effect of the toxin is curare-like, decreasing the ability of muscle to respond to direct stimulation. Heckly et al. (1960) showed that the principle site of systemic absorption is the small intestine. Indeed, medical dogma states that botulism occurs when botulinal toxin is absorbed from the gastrointestinal tract, circulates in the blood-stream, and is delivered to the site of action, which is, apparently, the acetylcholine release mechanism in the peripheral nervous system. However, Petty (1965) mentioned other possible ways in which the disease could result. For instance, if the organism could become established in the · ·ɔstinal tract, the toxin produced would be absorbed directly into the ɔd and lead to intoxication. There also exists the possibility that botulism could result from toxin produced by organisms lodged in tissues of the body by accidental puncture wounds. It is, however, widely accepted in the medical field that the site of action is on the presynaptic junction of the cholinergic synapses (Lewis and Cassel, Jr., 1964). In fact, unequivocal evidence of nerve-induced acetylcholine release from poisoned nerve muscle preparations was presented twenty years ago by Burgen et al. (1948).

The outbreaks of botulism in the period 1899-1949, as discussed by Osheroff et al. (1964), relate to a variety of home-processed foods. Death of victims is usually ascribed to suffocation due to paralysis of respiratory muscles. Specific antitoxins to each serotype of toxin, commercially produced in horses through injection of toxoid preparations, are available for specific treatment of intoxicated individuals. Bowmer (1963) gives the details of preparation and assay of the international

standards for types A, B, C, D, and E antitoxins.

In order to treat a suspected case of botulism with specific antitoxin, it is first necessary to determine both the type and quantity of toxin present in the suspect material. A very important consideration to be made in this context is the effect of the material used as diluent on the toxin and on the toxin-containing carrier. Type A toxin was shown by Buehler et al. (1947) to be a protein possessing the properties of a globulin, and so it is susceptible to various protein denaturing agents. For example, Spero (1958) showed that the toxin is inactivated by alkali; Cartwright and Lauffer (1958) noted that high temperatures could decrease toxicity; Boor et al. (1955) discussed toxin inactivation and potentiation by the effect of salts and colloids; Duff et al. (1956a) considered the effects of the buffer used as diluent. Clearly, assay procedures must be standardized in that a common and suitable diluent is used. This is requisite in order to give workers in this area a common basis for discussing results.

At the present time, suspect material is assayed in vivo using mice or guinea pigs; however, Cartwright and Lauffer (1952) reported the assay of toxin using goldfish. This method did not achieve widespread acceptance. Fifty per cent endpoints are determined based on per cent survivors and are analyzed according to the probit method of either Reed and Muench (1938) or Weiss (1948). The report by Schantz et al. (1958) stating that bioassay can underestimate true toxicity by sixty per cent stimulated even greater interest in the development of an in vitro assay method.

Early work in this area by Lamanna (1948) showed that both

crystalline and amorphous preparations of type A toxin were capable of causing hemagglutination of red blood cells of chickens, guinea pigs, rabbits, sheep, and man. This report caused considerable interest, not only because agglutination of red blood cells could be quantitated, and therefore, possibly used as an in vitro assay method, but also because it had always been thought that the toxin was a homogenous toxic protein. It was also shown that antitoxin specifically prevented the hemagglutination reaction. Lamanna and Lowenthal (1951) later showed that the hemagglutination caused by type A toxin was due to a component separable from the toxin. Since these same workers discovered that type B antitoxin is capable of neutralizing the hemagglutinating activity but not the toxicity of type A toxin, the possibility of its application as an assay method was discarded. Wagman (1954) determined that the agglutinin particles are very small nontoxic units which can be removed from the toxin without loss of toxicity. The advantages, limitations, and general applications of hemagglutination and hemagglutination-inhibition reactions are discussed by Stavitsky (1954).

Workers have continued studying the hemagglutination reaction of botulinal toxins. This interest stems partially from the possible role of the hemagglutinin in affecting results of in vivo as well as in vitro assays. Recent work with the system by DasGupta and Boroff (1967), showed that chromatographic isolation of hemagglutinin-free toxin was possible. However, Schantz (1968)* has determined that this 'pure'

*Schantz, E. J., United States Army Biological Laboratories, Fort Detrick, Maryland. The dissociation of hemagglutinin-free type A botulinum toxin. Private communication. 1968.

toxin becomes almost completely atoxic within a week. Thus, since 'pure' toxin is highly unstable, assays must be performed using toxin which is still part of the hemagglutinin-toxin complex elaborated by the organism.

Other possibilities for *in vitro* assay of type A toxin have been studied. Boroff and Fitzgerald (1958), for example, noted a relationship between toxicity and fluorescence of type A toxin, and determined that both of these properties of the toxin decrease with an increase in pH. However, Schantz et al. (1960) found that the specific region of the toxin molecule responsible for toxicity is not responsible for fluorescence. Thus, this system could not be used as the basis of an *in vitro* assay. Johnson et al. (1965) studied serological procedures which might be applicable to the detection of botulinal toxins in foods. However, this method is rather cumbersome and has proved to be of little practical importance.

Recently, Marshall and Quinn (1967) showed that type A toxin is capable of inhibiting the enzyme acetylcholinesterase *in vitro*, and also that the inhibition is detectable spectrophotometrically. Reports concerning the *in vitro* estimation of organic phosphate inhibitors of acetylcholinesterase are many and since type A toxin does inhibit acetylcholinesterase, spot tests based on the methods of Cook (1955a, 1955b) and Sandi and Wight (1961), for example, may be used to show enzyme inhibition qualitatively. However, techniques to quantitate the toxin are needed for an assay method. Also, to determine just how the inhibition is accomplished, kinetic studies must be done, preferably based on the methods given by Dixon and Webb (1964). Characteristics of the enzyme,

which were described by Wilson (1961) must be considered, as well as the physiological activity of the enzyme as discussed by Fatt and Katz (1951), Takeuchi and Takeuchi (1959), Burnstock and Holman (1961), Bell and Burnstock (1965), Boter and Ooms (1967), Karlin (1967), and Robinson and Bell (1967).

MATERIALS AND METHODS

Reagents

Crystalline type A botulinal toxin was kindly supplied by E. J. Schantz, United States Army Biological Laboratories, Fort Detrick, Maryland. This toxin was composed of both the hemagglutinin and toxin components. Purified crystalline antitoxin, types A, B, and E, was supplied by the Communicable Disease Center, Atlanta, Georgia. Bovine plasma albumin was supplied by Manuel Coria, National Animal Disease Laboratories, Ames, Iowa.

Lactic acid dehydrogenase (type III) was purchased from the Sigma Chemical Company, St. Louis, Missouri and its substrate, sodium lactate, from Fisher Laboratory Chemical, Fair Lawn, New Jersey. The enzymes, acetylcholinesterase (types I and III) and cholinesterase (type IV), as well as their substrate, acetylcholine chloride, were also obtained from Sigma. The chromogenic substrate of the esterases, indophenyl acetate (IPA), was obtained from Mann Research Laboratories, New York, New York. Nicotinamide adenine dinucleotide (NAD) was purchased from Calbiochem, Los Angeles, California, and Tris buffer, from Aldrich Chemicals, Milwaukee, Wisconsin.

Assay of Type A Botulinal Toxin

In vivo

The crystalline toxin was reconstituted and diluted for use in 0.9 per cent saline. One tenth ml of each dilution was used for

intraperitoneal injection of each of ten white Swiss mice, 18-22 g, obtained from the Midwest Animal Colonies, Corning, Iowa. Number of deaths was recorded over a four day period, and LD_{50} values were determined by the method of Weiss (1948) after three replications of the assay had been completed.

In vitro

Acetylcholinesterase inhibition by type A toxin Preliminary studies on this system were based on qualitative methods for detecting organic phosphate inhibitors of acetylcholinesterase activity on acetylcholine (Cook, 1955a, 1955b). The fact that inhibition of acetylcholinesterase occurs in vitro through the action of type A botulinum toxin was reported earlier by Marshall and Quinn (1967). The spectrophotometric methods and equipment used by these workers were also used in these studies. In order to characterize the type of inhibition involved in this system, a Lineweaver-Burk plot was accomplished using 1.00×10^{-5}M, 1.25×10^{-5}M, 1.50×10^{-5}M, 2.50×10^{-6}M, 5.00×10^{-6}M, and 7.50×10^{-6}M concentrations of IPA. The reaction mixture was composed of 10^{-8}mg/ml (0.3 LD_{50}/ml) type A botulinum toxin, IPA, and type III acetylcholinesterase at a final concentration of 0.25 μM units/ml. Eight replications of the data for the plot were made, and the individual slopes in each replication were obtained by averaging eight values over the entire chart since the velocity did not change in the time interval used (60 min), i.e., each subsequent value was identical, excepting for experimental error, to that of the initial velocity. The values obtained for velocity varied less than one per cent and so were

averaged to give the greatest level of precision obtainable.

The gradient of inhibition produced by the toxin was tested using 2.50×10^{-6}M IPA, type III acetylcholinesterase, and varying concentrations of botulinum toxin. Each assay was run in quadruplicate and the final optical density value was used for extrapolation to zero in order to demonstrate the gradient of inhibition which occurs due to the toxin. A dose-response curve was constructed using fifteen concentrations of toxin to determine the applicability of this inhibition system for assay of the toxin *in vitro*.

Specificity of inhibition for biologically active toxin To determine the specificity of the inhibition reaction for active type A botulinum toxin, type A antitoxin was diluted to titer and added to the toxin. This mixture was incubated at 37C for 30 min before addition of type III acetylcholinesterase. Inactivation of the toxin (at an initial potency of $3 \times 10^6 LD_{50}$/ml) was accomplished by three alternate procedures, namely (a) by alkali treatment (0.01N NaOH, pH 12, 3 hr); (b) by boiling the toxin for 15 min; and (c) by 3 months' storage in 0.9 per cent NaCl, pH 7.2, at 4C under air atmosphere. To be sure that following such treatments botulinum toxin was indeed inactive, 10 white Swiss mice, 18-22 g, were injected with both (a) the equivalent of $3 \times 10^6 LD_{50}$ doses of treated toxin and (b) a similar dose of untreated toxin. The surviving animals were observed for 7 days.

Specificity of inhibition for type III acetylcholinesterase Assays were run, based on the procedures used previously with type III acetylcholinesterase, using 0.25 μM units/ml final concentration of

acetylcholinesterase (type I) and 0.25 μM units/ml final concentration of cholinesterase (type IV) to test the effect of type A botulinal toxin on these systems.

Lactic acid dehydrogenase (type III), 0.0001 mg/ml final concentration, was also used in these studies and was assayed using the method of Ames, et al. (1963). These assays were run on a two-channel Beckman DB recording spectrophotometer after incubating the toxin and enzyme at room temperature for 0 min, 10 min, 20 min, and 30 min. Control assays were run using (a) sodium lactate, 5 mg/ml and the enzyme to test for activity, and (b) sodium lactate, 5 mg/ml, with the appropriate dilution of toxin to test for possible changes in optical density in the absence of the enzyme.

The dilutions of toxin used in each of these assays were 10^{-2} mg/ml through 10^{-9} mg/ml. Two duplications of each assay were run.

Assay of type A botulinum toxin using acetylcholinesterase inhibition To develop the system for in vitro assay of type A toxin, ten doses of the toxin were chosen for further study, i.e., 1×10^{-10} mg/ml (0.003 LD_{50}/ml), 2×10^{-10} mg/ml (0.006 LD_{50}/ml), 1×10^{-9} mg/ml (0.03 LD_{50}/ml), 1×10^{-8} mg/ml (0.3 LD_{50}/ml), 2×10^{-8} mg/ml (0.6 LD_{50}/ml), 5×10^{-8} mg/ml (1.5 LD_{50}/ml), 1×10^{-7} mg/ml (3 LD_{50}/ml), 1×10^{-6} mg/ml (30 LD_{50}/ml), 2×10^{-6} mg/ml (60 LD_{50}/ml), and 1×10^{-5} mg/ml (300 LD_{50}/ml). Each dose was characterized, using 2.50×10^{-6} M IPA and 0.25 μM units/ml of type III acetylcholinesterase, by the methods given by Marshall and Quinn (1967). However, in these experiments, the toxin and enzyme were incubated together for 20 min prior to IPA addition and the course of

each inhibition curve was charted for 100 min. Each dose was used in twenty separate experiments and after the charting was completed, the slope of each curve was determined using the value between 40 min and 60 min on the chart. Statistical analysis of the data was made using conventional methods (Finney, 1952, Steel and Torrie, 1960).

In order to determine the ability of the assay to detect and quantitate toxin in food samples, assays were run, based on normal procedures, using hamburger and pea broth as vehicles for toxin. Broths were prepared by boiling 50 g hamburger in 100 ml tap water and 10 g peas in 50 ml tap water. Both samples were boiled for 5 min. The suspended material in each sample was allowed to settle for 8 hr before toxin dilutions were added. Final concentrations of toxin used in the two food broths were 1×10^{-8}mg/ml, 1×10^{-7}mg/ml, 2×10^{-6}mg/ml, and 1×10^{-5}mg/ml. All assays were run in duplicate.

RESULTS AND DISCUSSION

Assay of Type A Botulinal Toxin

In vivo

The botulinal A toxin had a potency of 3 x 10^{10} mouse LD_{50}/g. The variability over three replications was very small, indicating that maximal precision was obtained in determining the estimate of toxin potency.

In vitro

Acetylcholinesterase inhibition by type A toxin Spot tests performed in an attempt to show inhibition of type III acetylcholinesterase by the toxin indicated that such inhibition was occurring in the system. In fact, using this qualitative approach, it was possible to detect as little as $10^{-4}LD_{50}$ of toxin. This method could be used as a means for the detection of toxin in foods or other types of carrier materials; however, qualitative techniques, although useful, cannot serve as the basis for an assay procedure. Therefore, the same enzyme, type III acetylcholinesterase, was used with the chromogenic substrate in the development of spectrophotometric analyses.

There was a definite and reproducible inhibition of type III acetylcholinesterase activity by the botulinal toxin appearing as a difference in optical density values in test versus control readings over the time interval specified (Table 1). Indeed, the standard slopes of the inhibition curves, obtained directly, were significantly different. The

Table 1. Final optical density values at 625 mμ obtained at specified time intervals by use of indophenyl acetate (IPA), acetylcholinesterase (Ac'ase, type III), types A, B, and E antitoxin, and type A botulinum toxin in enzyme inhibition studies (Marshall and Quinn, 1967)

Reaction mixture components and concentrations[a]	OD	Time (min)	Difference OD units[b]
Ac'ase[c] + IPA + 3.3 x 10^{-6}mg of toxin	.409	48	---
Ac'ase + IPA	.560	48	.151
Ac'ase + IPA + 3.3 x 10^{-6}mg of toxin + A antitoxin[d]	.564	48	---
Ac'ase + IPA	.560	48	.004
Ac'ase + IPA + 3.3 x 10^{-6}mg of toxin + A antitoxin[e]	.517	48	---
Ac'ase + IPA	.560	48	.043
Ac'ase + IPA + 3.3 x 10^{-6}mg of toxin + B antitoxin	.441	48	---
Ac'ase + IPA	.560	48	.119

[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 units/ml (0.25 μM unit/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 25C before being added to the reaction mixture.

Table 1. (Continued)

Reaction mixture components and concentrations[a]	OD	Time (min)	Difference OD units[b]
Ac'ase + IPA + 3.3 x 10^{-6}mg of toxin + E antitoxin	.426	48	---
Ac-ase + IPA	.560	48	.134
Ac'ase + IPA + 3.3 x 10^{-10}mg of toxin	.457	48	---
Ac'ase + IPA	.560	48	.103
Ac'ase (0.5 /ml) + IPA + 3.3 x 10^{-10}mg of toxin	.382	120	---
Ac'ase (0.5 /ml) + IPA	.560	120	.178
Ac'ase + IPA + 3.3 x 10^{-10}mg of toxin + A antitoxin	.551	48	---
Ac'ase + IPA	.560	48	.009
Ac'ase + IPA + 3.3 x 10^{-10}mg of toxin + B antitoxin	.460	48	---
Ac'ase + IPA	.560	48	.100
Ac'ase + IPA + 3.3 x 10^{-10}mg of toxin + E antitoxin	.458	48	---
Ac'ase + IPA	.560	48	.102

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 incubation of homologous antitoxin with toxin before enzyme addition 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 (types B and E) at either toxin concentration and through the partial release of inhibition by addition of homologous antitoxin after enzyme addition. In fact, the serological cross-reaction observed by Lamanna and Lowenthal (1951) between type A toxin and type B antitoxin obviously did not occur in this system. Although type III acetylcholinesterase inhibition is reproducible and constant for given toxin, enzyme, and substrate concentrations, enzyme inhibition is never complete.

It was found that the difference in optical density (amount of inhibition of the enzyme by the toxin) could be increased by keeping the enzyme and substrate concentrations constant while increasing the toxin concentration, or by decreasing the enzyme concentration and increasing the incubation time. Also, when toxin A and antitoxin A were incubated for 10 min prior to the addition of enzyme 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; that is, about half the

optical density difference was observed relative to that in the toxin-enzyme system incubated for only 10 min. From these data, it was speculated that there may be an action of type III 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 toxin fragments not binding to antitoxin and whose concentration would be related to the time of incubation or of exposure of toxin to the enzyme molecule. The data in Table 1 indicate that some system may be causing a potentiation of toxicity such that the amount of antitoxin required for neutralization is no longer sufficient to prevent enzyme inhibition. The preceding discussion was taken from Marshall and Quinn (1967).

As mentioned previously, inhibition of type III acetylcholinesterase activity by type A botulinum toxin was only partially reversed by addition to the system of type A antitoxin which had been diluted to titer, whereas the addition of heterologous antitoxins did not produce even a partial reversal of the toxin-induced inhibition. Therefore, the enzyme inhibition by the toxin is due only to the toxic portion of the toxin molecule and not to enzyme involvement with hemagglutinin (Lamanna, 1948, Lamanna and Lowenthal, 1951).

The inhibition response pattern noted (Table 1, Figure 1) would be typical of the 'mixed' type of inhibition of the enzyme (Dixon and Webb, 1964), as only a proportion of the toxin activity is susceptible to neutralization by specific antitoxin after toxin-enzyme interaction. This observation was validated by the Lineweaver-Burk plot (Figure 2)

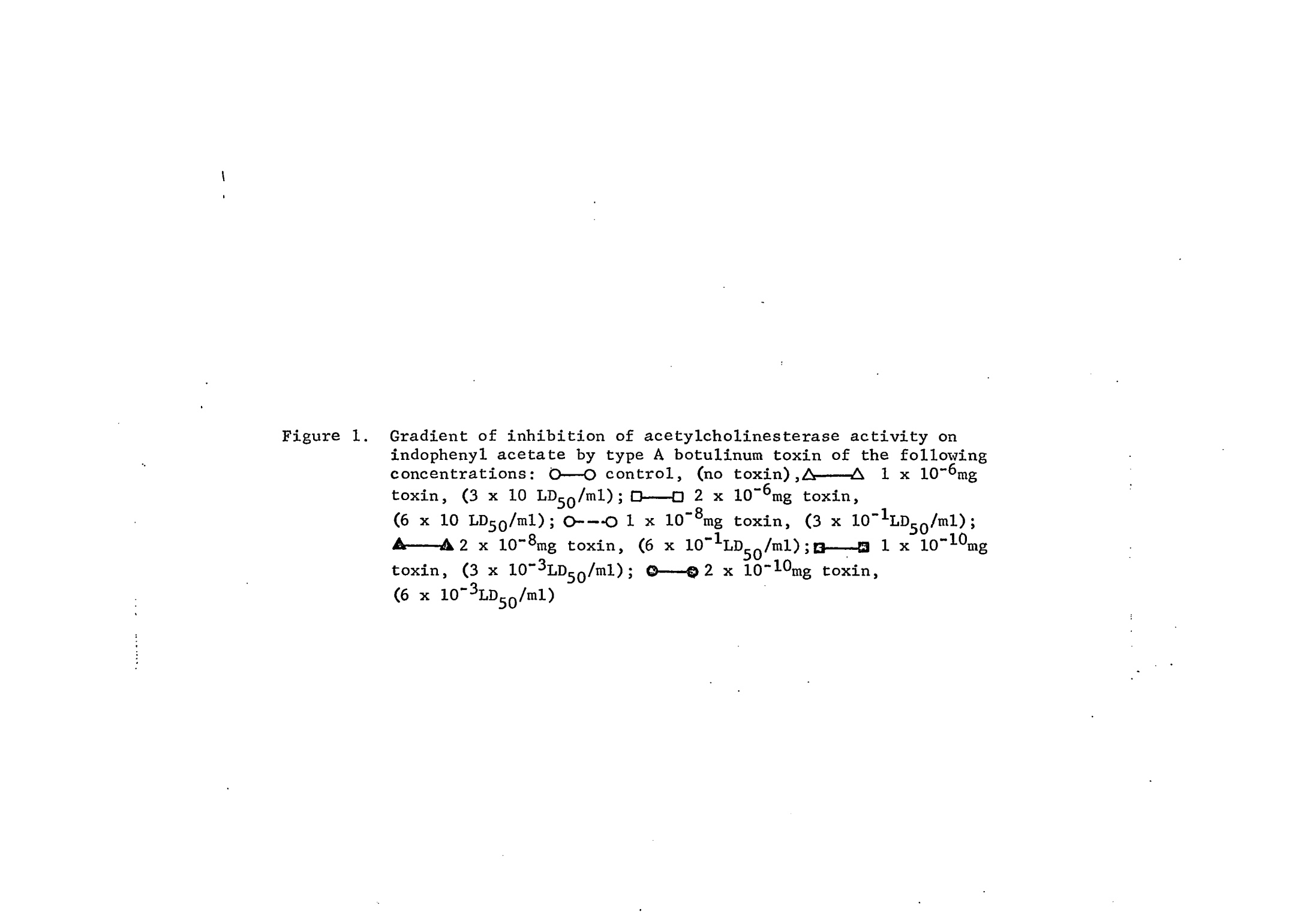


Figure 1. Gradient of inhibition of acetylcholinesterase activity on indophenyl acetate by type A botulinum toxin of the following concentrations: O—O control, (no toxin), △——△ 1 x 10^{-6}mg toxin, (3 x 10 LD_{50}/ml); □——□ 2 x 10^{-6}mg toxin, (6 x 10 LD_{50}/ml); O- -O 1 x 10^{-8}mg toxin, (3 x $10^{-1}$$LD_{50}$/ml); ▲——▲ 2 x 10^{-8}mg toxin, (6 x $10^{-1}$$LD_{50}$/ml); ⊡——⊡ 1 x 10^{-10}mg toxin, (3 x $10^{-3}$$LD_{50}$/ml); ⊙——⊙ 2 x 10^{-10}mg toxin, (6 x $10^{-3}$$LD_{50}$/ml)

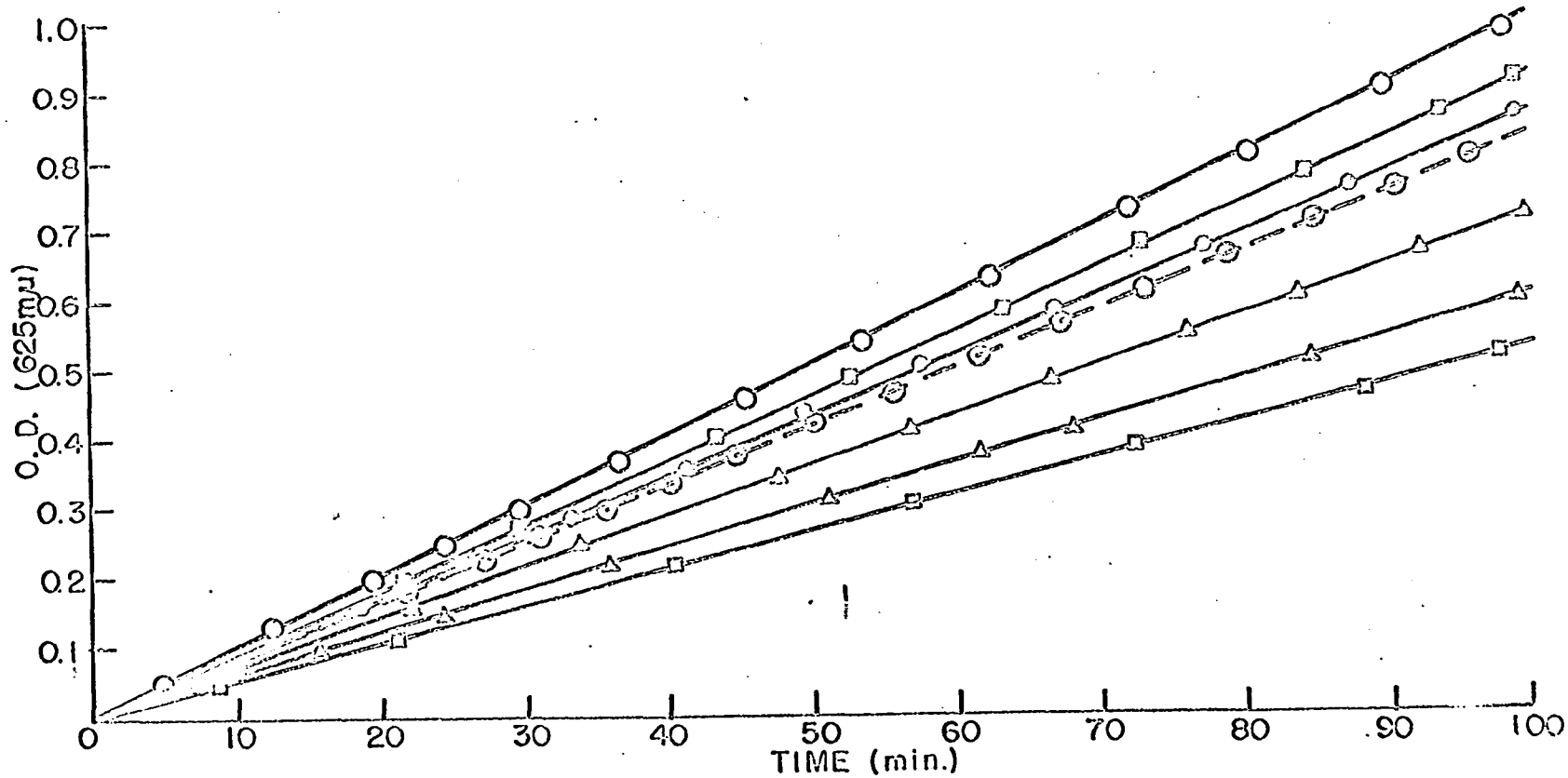

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D. (625mμ)
0
10
20
30
40
50
60
70
80
90
100
TIME (min.)

Figure 2. Lineweaver-Burk plot of the interaction between type III acetylcholinesterase (0.25 μM units), indophenyl acetate (2.5×10^{-6} M), and type A botulinum toxin (0.3 LD_{50}/ml)

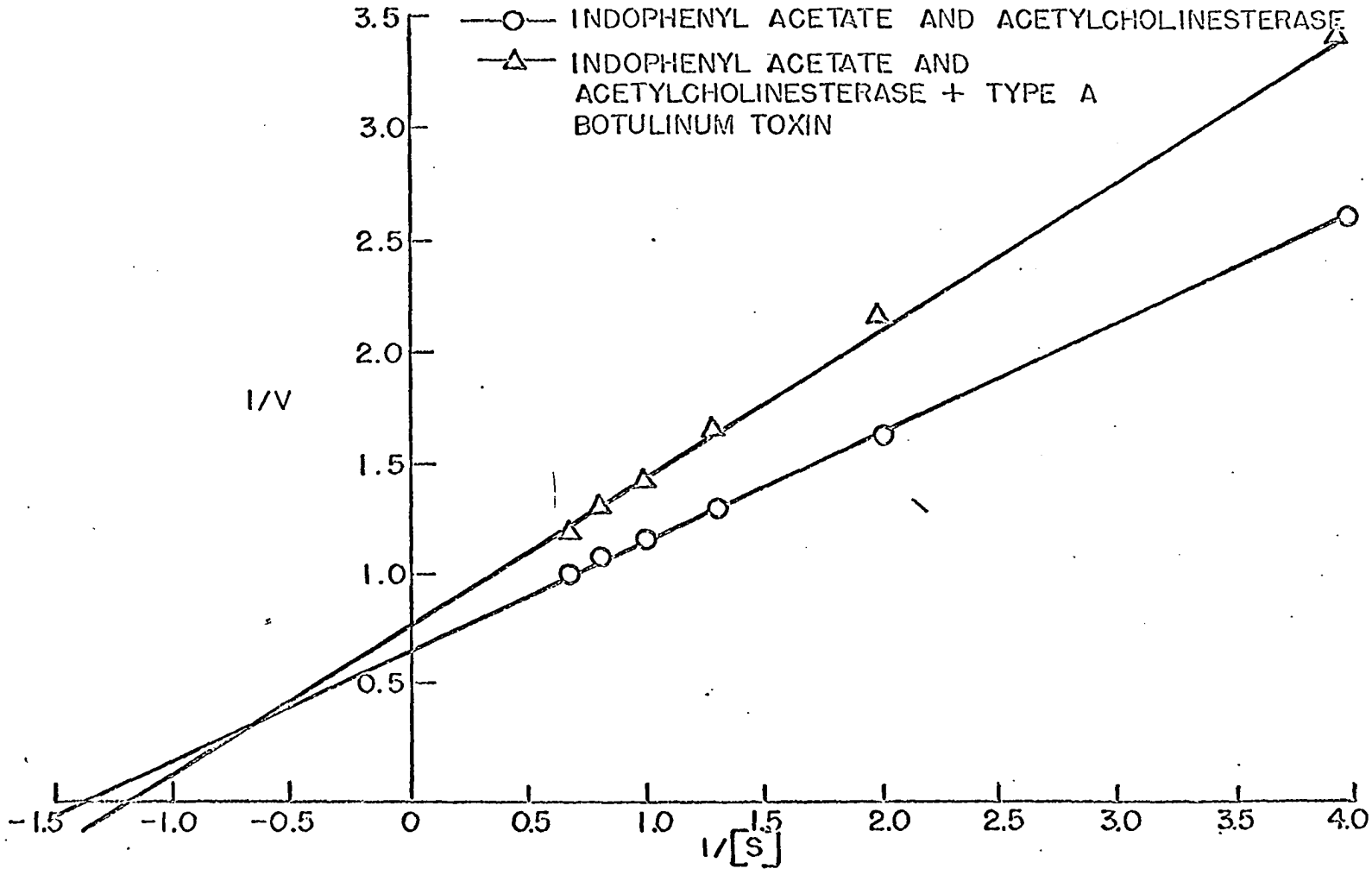

INDOPHENYL ACETATE AND ACETYLCHOLINESTERASE
INDOPHENYL ACETATE AND ACETYLCHOLINESTERASE + TYPE A BOTULINUM TOXIN
1/V
1/[S]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

Table 2. Data for the Lineweaver-Burk plot of the action of acetylcholinesterase (0.25 μM units/ml) with and without type A botulinum toxin (10^{-8}mg/ml; $0.3LD_{50}$/0.1 ml) on indophenyl acetate (2.5×10^{-6}M) at 22C using light of 625 mμ

$\{S\}$ (IPA)[a]	$1/\{S\}$[b]	$1/V$[c] (with toxin)	$1/V$[d] (without toxin)
1.50×10^{-5}M	0.67	1.20	1.00
1.25×10^{-5}M	0.80	1.32	1.07
1.00×10^{-5}M	1.00	1.45	1.15
7.50×10^{-6}M	1.33	1.65	1.35
5.00×10^{-6}M	2.00	2.15	1.60
2.50×10^{-6}M	4.00	3.40	2.60

[a]Molar concentration of IPA.

[b]Reciprocal of molar concentration of IPA coded by deleting 10^{5}M from each concentration.

[c]Reciprocal of slope of inhibition curve at designated IPA concentrations (averaged values of 8 replications).

[d]Reciprocal of slope of IPA and enzyme curves at designated IPA concentrations (averaged values of 8 replications).

which was based on the values in Table 2. The partial inhibition of acetylcholinesterase by botulinum toxin in the presence of antitoxin specific for the toxin serotype, is better explained by the reversible nature of a part of the enzyme inhibition which is characteristic of the mixed type of inhibition, than by the fragmentation of toxin to give nonantigenic but toxic fragments as was speculated earlier (Marshall and Quinn, 1967). Complete inhibition of the enzyme activity by

botulinum toxin would not be possible in this system regardless of the concentration of toxin used, and complete enzyme inhibition was never obtained experimentally. A complete lack of inhibition of the enzyme is obtained only when the toxin and homologous antitoxin are incubated before enzyme addition.

As was seen in Figure 1, there is a gradual decrease in enzyme inhibition produced by decreasing the concentration of botulinum toxin. The fact that this gradient occurs consistently and that the slopes and the final inhibition values for any given concentration of toxin are highly reproducible indicated the value of this method for *in vitro* assay of type A botulinum toxin. Indeed, a dose-response curve was constructed using fifteen concentrations of the toxin. Table 3 gives the actual doses in mg/ml and the LD_{50} doses/ml which are equivalent to the transformed abscissa values. The dose-response curve is given in Figure 3. In Table 4 the slope values at each dose level are found. Since monotonocity is a strict requirement for the dose-response curve in a valid assay situation, the linear portion of the curve at the lower dose levels only (10^{-10} mg/ml through 10^{-5} mg/ml) was used for development of the assay.

Specificity of inhibition for biologically active toxin Those mice injected with inactivated toxin preparations showed, over the course of the observation period, a complete lack of those typical symptoms which occur after the injection of active toxin, while control animals, injected with similar size doses of untreated toxin, died within an hour. It was shown that those mice injected with the storage

Table 3. Concentration of type A botulinum toxin in actual dose, transformed dose, and LD_{50} dose used in the construction of dose-response curves

Actual dose mg/ml	=	Transformed dose[a]	=	LD_{50} dose/ml
1×10^{-10}		0		3×10^{-3}
2×10^{-10}		0.301		6×10^{-3}
1×10^{-9}		1		3×10^{-2}
1×10^{-8}		2		3×10^{-1}
2×10^{-8}		2.301		6×10^{-1}
5×10^{-8}		2.699		1.5
1×10^{-7}		3		3
1×10^{-6}		4		30
2×10^{-6}		4.301		60
1×10^{-5}		5		3×10^{2}
1×10^{-4}		6		3×10^{3}
1×10^{-3}		7		3×10^{4}
1×10^{-2}		8		3×10^{5}
1×10^{-1}		9		3×10^{6}
1		10		3×10^{7}

[a] $(\log_{10}$ dose mg/ml) + 10.

Figure 3. Dose-response curve constructed using slope determinations after assaying fifteen concentrations of type A botulinal toxin using acetylcholinesterase inhibition

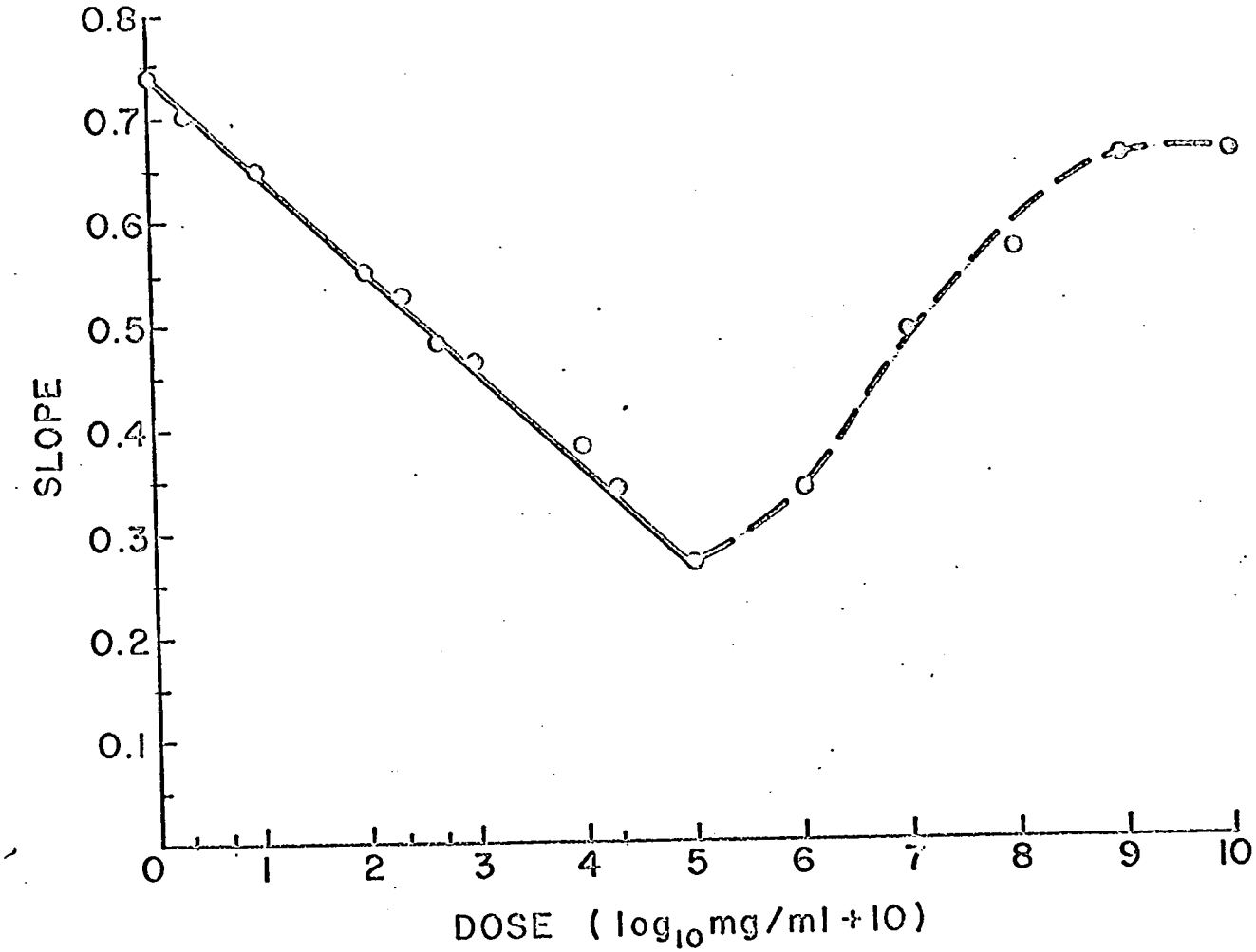

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
SLOPE
0
1
2
3
4
5
6
7
8
9
10
DOSE (log10 mg/ml + 10)

Table 4. Slope values from in vitro assays of the inhibition of acetylcholinesterase using fifteen dose levels of type A botulinum toxin

Toxin log dose + 10	Slope
0	.78,.72,.79,.71,.70,.80,.70,.71,.77,.72,.71,.76,.75,.83,.70,.81,.69,.70,.68,.78
0.301	.68,.74,.70,.67,.73,.70,.70,.62,.65,.71,.75,.71,.68,.77,.74,.70,.67,.74,.71,.70
1.0	.64,.68,.66,.72,.62,.68,.62,.63,.64,.70,.61,.64,.68,.62,.70,.60,.63,.64,.61,.66
2.0	.56,.56,.60,.58,.56,.61,.48,.58,.59,.48,.46,.48,.58,.58,.55,.49,.58,.59,.53,.48
2.301	.60,.61,.51,.52,.60,.50,.50,.46,.52,.60,.52,.52,.48,.53,.50,.52,.50,.53,.52,.50
2.699	.51,.54,.50,.48,.52,.56,.48,.46,.48,.49,.52,.46,.48,.47,.46,.51,.48,.50,.40,.51
3	.46,.41,.53,.46,.40,.53,.48,.45,.43,.43,.54,.52,.40,.44,.52,.55,.43,.46,.42,.43
4	.38,.36,.36,.44,.42,.34,.36,.36,.39,.40,.44 .36,.37,.33,.38,.41,.37,.38,.44,.36
4.301	.34,.36,.25,.35,.33,.32,.36,.32,.38,.35,.38,.28,.36,.38,.34,.35,.36,.30,.28,.38
5	.32,.26,.28,.22,.30,.24,.30,.24,.33,.26,.20,.32,.27,.25,.29,.30,.26,.28,.30,.27
6	.35,.29,.36,.38,.37,.33,.35,.32,.37,.29,.35,.39,.33,.35,.36,.31,.30,.37,.33,.29
7	.46,.50,.41,.43,.49,.52,.56 .52,.50,.46,.49,.48,.50,.43,.49,.53,.56,.52,.50,.49
8	.65,.52,.58,.63,.61,.57,.58,.53,.59,.58,.59,.60,.58,.63,.54,.56,.55,.51,.58,.56
9	.60,.68,.73,.70,.65,.69,.65,.61,.72,.70,.68,.69,.61,.63,.64,.62,.63,.67,.68,.70
10	.68,.70,.69,.68,.64,.71,.60,.68,.65,.68,.60,.60,.68,.67,.66,.71,.72,.68,.60,.61

inactivated toxin preparation were able to withstand injection of 30 LD_{50} doses of active toxin after a two-week rest period, which indicated that this preparation was acting as a toxoid. Injection of heat-inactivated and alkali-inactivated toxin preparations did not protect mice injected with 30 LD_{50} doses of active toxin after a two-week rest period. Since the dosage of toxin injected was so great, it can be said that a complete loss of the biological activity of the toxin must have been produced by the various inactivation techniques. In vitro toxin assays were made using toxin more dilute than that used in the in vivo test so that a complete lack of biological potency of the toxin could be assumed if mice were able to survive the test.

Using toxin inactivated by the various methods, employing normal procedures for the assay, and comparing the slope and final optical density values after a specified time interval (100 min), it was seen that the slopes of the resultant plots was exactly that of the IPA plus enzyme controls. In the positive controls, untreated toxin inhibited the enzyme in the expected manner. This finding has interesting physiological implications since it indicates that botulinum toxin must be in a biologically active form in order to inhibit the action of the enzyme in vitro; in other words, toxoid preparations apparently have no effect on the enzyme. Studies were not done, however, to test for in vivo enzyme inhibition. A recent study by Malaney and Davis (1967) has shown that acetylcholinesterase inhibition due to micropollutants in drinking water produces symptoms in humans referrable to the nervous system. This finding gives some credance to the idea that some of the

symptoms seen in botulism intoxication could be due to the inhibition of acetylcholinesterase. However, in vivo inhibition of this enzyme by the toxin has not been demonstrated experimentally.

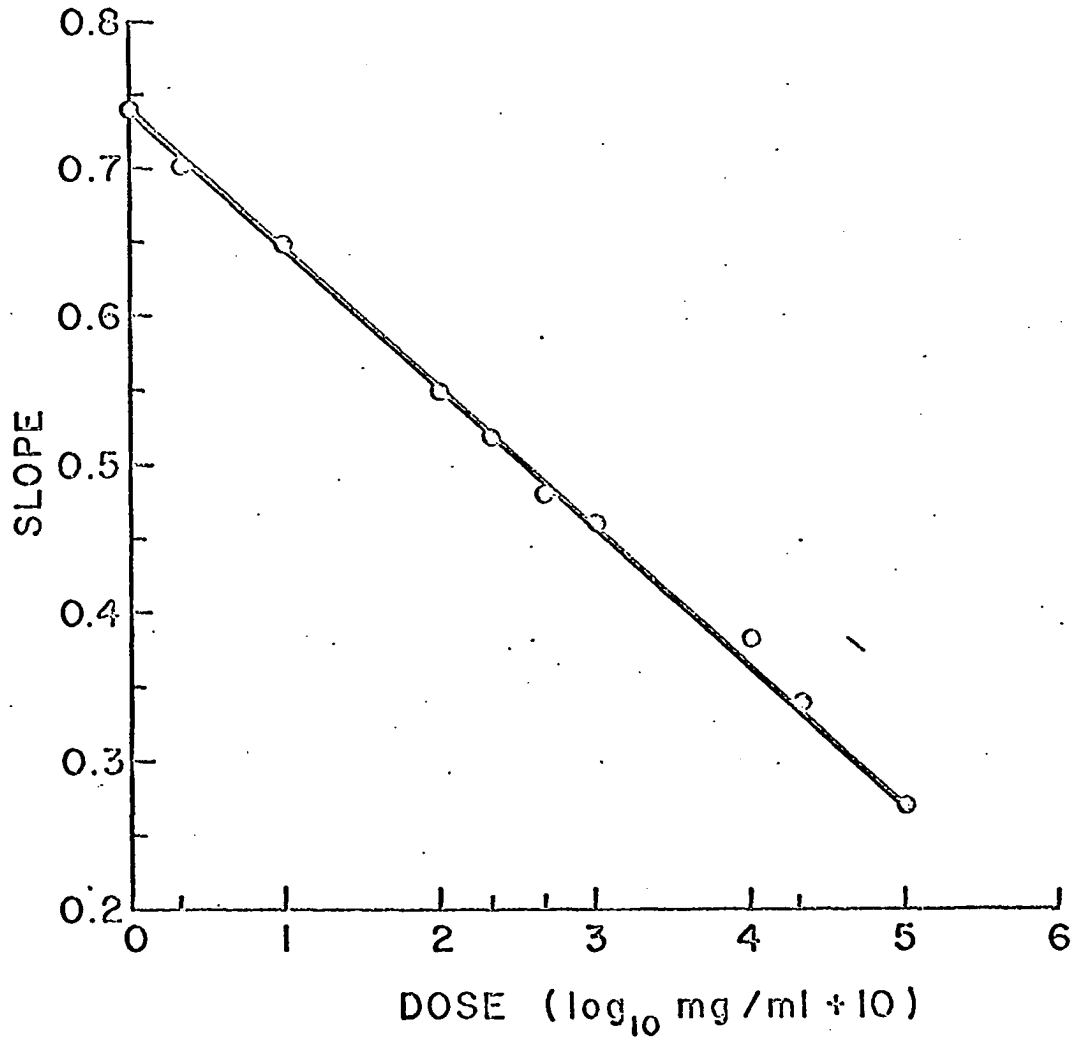
Specificity of inhibition for type III acetylcholinesterase Assays which were run using type I acetylcholinesterase and type IV cholinesterase indicated that the typical toxin-induced enzyme inhibition which is seen with type III acetylcholinesterase does not occur as such in these systems. A slight amount of inhibition was noted using type I acetylcholinesterase; however, at the enzyme and substrate concentrations used, the slope of the inhibition curves was less than 0.1 unit below the control curves regardless of toxin concentration. The IPA plus enzyme controls were typical of those seen with type III acetylcholinesterase. The nonspecific type IV cholinesterase used in these studies was not inhibited by the toxin at the enzyme-substrate-toxin concentrations used. It can be concluded from these results that neither of these systems could greatly influence the results of in vitro assays using type III acetylcholinesterase with suspect material containing these other esterases. The studies performed using lactic acid dehydrogenase showed a complete lack of enzyme inhibition by the toxin, and therefore, the presence of this enzyme in suspect material would not affect in vitro assay of the toxin using the acetylcholinesterase-IPA system.

Unfortunately, it cannot be concluded that the assay system developed from the inhibition of type III acetylcholinesterase on the chromogenic substrate will be impervious to either potentiation or

negation of typical inhibition curves. It is quite possible that there exists an enzyme or a material of some nature in suspect material which could affect the results of the inhibition of enzyme activity by the toxin. However, it is not possible to test this likelihood without extensive practical application of the assay itself.

Assay of type A botulinum toxin using acetylcholinesterase inhibition The linear curve which was established by the use of the toxin-induced enzyme inhibition is given in Figure 4. Only those concentrations of toxin between 10^{-10}mg/ml and 10^{-5}mg/ml can be quantitated using this method. As can be seen in Figure 3, concentration effects and unexplained interferences in the system cause an increase in slope (decreased inhibition of enzyme) above toxin concentrations of 10^{-5}mg/ml. Thus, the assay must be restricted to dose levels below this amount. A possible explanation for the concentration effects may be the polymerization or aggregation of the toxin molecules at concentrations above 10^{-5}mg/ml. That this phenomenon does occur with proteins has been discussed, for example, by Kuff et al. (1955) and by Thomson and Moss (1956). It is a well-accepted fact that proteins and globulins will aggregate or polymerize if these materials are present in solution in high concentration. If this phenomenon occurs with concentrations of toxin above 10^{-5}mg/ml, the decreased inhibition could be due to a masking of the site(s) on the toxin molecule which interacts with and inhibits type III acetylcholinesterase activity. In this way, then, the change of slope seen in Figure 3 could be explained. However, it must be mentioned that the range of effectiveness of this assay is in the area of interest for

Figure 4. Dose-response curve of botulinum A toxin using acetylcholinesterase (type III) inhibition


0.8
0.7
0.6
0.5
0.4
0.3
0.2
SLOPE
0
1
2
3
4
5
6
DOSE (log10 mg/ml + 10)

quantitation studies. Dilutions of suspect material must also be made using in vivo assay techniques in order that an endpoint may be reached.

The analysis of variance of six of the doses used for the assay is given in Table 5. This analysis was performed using only the six equally-spaced doses from 10^{-10} mg/ml through 10^{-5} mg/ml in order to simplify the statistical methods. It is obvious that there is a significant difference in the response to the treatments and also that the bulk of the variation among the contrasts can be explained by linear regression. Since the quadratic, cubic, quartic, and quintic comparisons are not significant, a straight line may be drawn through the points to characterize the enzyme inhibition; the slope of the regression line was calculated to be -0.91.

Table 5. Analysis of variance on the slope determinations at six equally-spaced doses

Source	Df	SS	MS	F[a]
Treatments	5	2.9506	0.5901	295.05
linear	(1)	2.9459	2.9459	1472.95
quadratic	(1)	0.0000005	0.0000005	0.00025
cubic	(1)	0.0021	0.0021	1.05
quartic	(1)	0.0024	0.0024	1.2
quintic	(1)	0.0002	0.0002	0.10
Within treatments	114	0.1971	0.002	

[a]Tabular $F_{114}^{5} = 2.29$ at 0.05 Tabular $F_{114}^{1} = 3.92$ at 0.05.

**Significant

Assays made on hamburger and pea broth using the final assay procedure showed that salts and other food materials from these samples did not interfere with the typical inhibition response (Table 6). The assay procedure enabled quantitation of toxin in these two food samples, and will provide a means for detecting and quantitating type A botulinum toxin.

Table 6. Assay for type A botulinal toxin using hamburger and pea broths as vehicles for toxin

Toxin concentration mg/ml	Hamburger broth				Pea broth			
	$1x10^{-8}$	$1x10^{-7}$	$2x10^{-6}$	$1x10^{-5}$	$1x10^{-8}$	$1x10^{-7}$	$2x10^{-6}$	$1x10^{-5}$
Slope	.54	.48	.34	.27	.55	.48	.36	.28
Values	.56	.46	.34	.30	.57	.44	.32	.26

SUMMARY

In vitro assay of the A serotype of botulinum toxin using a system of enzyme inhibition has been discussed. The assay procedure, given below, has enabled quantitation of toxin.

1) Set recording spectrophotometer to read 625 mu wavelength transmission and equilibrate the sample holder temperature to 22C
2) Incubate 0.1 ml toxin and 0.1 ml enzyme (25 uM units/ml) together for 20 min
3) Add 0.1 ml IPA (2.5×10^{-4}M) to the toxin-enzyme mixture and dilute with Clark-Lubs buffer, pH 8.0, to 10 ml
4) Set up controls using IPA + buffer, as well as IPA + enzyme + buffer (the final volume in all four flasks should be 10 ml)
5) Transfer 3 ml of each reaction mixture to a spectrophotometer cuvette with a 1 cm light path, and begin to chart the reaction
6) Allow 60 min to elapse before the reaction is stopped
7) Determine the slope of the curve (for the system toxin-enzyme-IPA) as well as that of the IPA + enzyme control, by measuring the slope value established during the 50-60 min interval on the absorbance trace given on the recorder chart
8) Determine the toxin concentration in the unknown by comparing this slope value with those given in Table 4

Although this *in vitro* assay method has been shown to be effective when using purified reagents, its worth in a practical sense has yet to

be evaluated. Many analytical problems may arise. For example, how can one be sure that enzyme inhibition is due to toxin alone and not to some other material present in the toxin-containing sample? Can one be sure that a lack of enzyme inhibition is due to the absence of the toxin, or is it perhaps due to an interference of some nature with toxin activity? Will this assay give the same results when either 'pure' or crude toxin is used or when purified C. botulinum hemagglutinin is used? The potential areas of study originating with the development of this assay are many.

More work is needed to clarify the kinetics of the decrease in acetylcholinesterase inhibition by the toxin above a concentration of 10^{-5}mg/ml. The fact that decreased inhibition occurs is obvious, but the reason for this decrease is not. What exact effect does the toxin have on the enzyme? There is much work left to be done on this system especially considering the practical applications. As such, however, the system will enable a precise and rapid assay method at least for purified toxin preparations in laboratory situations.

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APPENDIX

Table 7. Mouse assay of type A botulinum toxin using the method of Weiss (1948)

Dose toxin mg/ml	$\log_{10}$(mg/ml)	Rep. I Survivors / No. tested	Rep. I Probit	Rep. II Survivors / No. tested	Rep. II Probit	Rep. III Survivors / No. tested	Rep. III Probit
3×10^{-1}	-0.52	0/10	3.22	0/10	3.22	0/10	3.22
3×10^{-2}	-1.52	0/10	3.22	0/10	3.22	0/10	3.22
3×10^{-3}	-2.52	0/10	3.22	0/10	3.22	0/10	3.22
3×10^{-4}	-3.52	0/10	3.22	0/10	3.22	0/10	3.22
3×10^{-5}	-4.52	0/10	3.22	0/10	3.22	0/10	3.22
3×10^{-6}	-5.52	2/10	4.16	3/10	4.48	2/10	4.16
3×10^{-7}	-6.52	5/10	5.00	5/10	5.00	5/10	5.00
3×10^{-8}	-7.52	10/10	7.14	10/10	7.14	10/10	7.14
3×10^{-9}	-8.52	10/10	7.14	10/10	7.14	10/10	7.14
0	----	10/10	7.14	10/10	7.14	10/10	7.14

$M = \log_{10} LD_{50}/mg = -6.52$

SE $\log_{10} LD_{50}/mg = 0.174$

$M_L = -6.52 + (-0.174) = -6.694$

$M_H = -6.52 + (+0.174) = -6.346$

Relative potency estimators:

R_L = antilog M_L = 2×10^7 LD_{50}/mg

R = antilog M = 3×10^7 LD_{50}/mg

R_H = antilog M_H = 4.6×10^7 LD_{50}/mg

Table 8. Slope values from assays made using type I acetylcholinesterase (ACE-I), type IV cholinesterase (CE-IV), and type III acetylcholinesterase (ACE-III) with IPA to determine the effect of type A botulinum toxin on enzyme activity (slope values determined using the interval from 40 min to 60 min on the Gilford chart)

Toxin mg/ml	ACE-I		CE-IV		ACE-III	
1×10^{-2}	0.85	0.87	0.83	0.89	0.59	0.60
1×10^{-3}	0.86	0.86	0.87	0.85	0.50	0.49
1×10^{-4}	0.76	0.79	0.85	0.82	0.34	0.37
1×10^{-5}	0.73	0.82	0.85	0.79	0.27	0.22
1×10^{-6}	0.80	0.76	0.86	0.81	0.38	0.38
1×10^{-7}	0.80	0.73	0.81	0.85	0.43	0.46
1×10^{-8}	0.75	0.77	0.80	0.79	0.55	0.54
1×10^{-9}	0.72	0.80	0.82	0.83	0.65	0.62
0	0.86	0.81	0.84	0.85	0.86	0.85

Table 9. Effect of type A botulinum toxin on the activity of lactic acid dehydrogenase after incubating the enzyme and toxin for varying time intervals

Toxin mg/ml	Optical density							
	0 min		10 min		20 min		30 min	
1×10^{-2}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-3}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-4}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-5}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-6}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-7}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-8}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
1×10^{-9}	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
control[a]	0	0	0	0	0	0	0	0
control[b]	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7

[a]Assays run without enzyme.

[b]Assays run without toxin.