THE REMOVAL OF BOTULINUS TOXIN FROM WATER

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THE REMOVAL OF BOTULINUS TOXIN FROM WATER

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I. PURPOSE

During the past war, crystalline botulinus toxin, type A was developed as a bacterial warfare weapon. Statements have been made that the use of botulinus toxin as a weapon could be as effective as an Atom Bomb as far as destruction of humans is concerned.

Many methods of dissemination are possible such as dusting over heavily populated areas and contaminating water reservoirs and water distribution systems.

Inasmuch as there is little data available on the destruction of botulinus toxin in water an exploratory study of chemical and physical methods of destruction appeared advisable (1). If a chemical method of destruction could be evolved that would fit into a water treatment process, preventive measures would be possible in case of enemy attack.

II. REVIEW OF THE LITERATURE

The crystallization and isolation of type A toxin was accomplished simultaneously and independently by two different groups of workers. Lamanna et. al. (2,3) reported crystalline toxin with an LD_{50} of 239.9×10^6 per milligram of nitrogen. Toxin prepared by Abrams et. al. (4) contained 220×10^6 M.L.D. per milligram of nitrogen. Both toxins, prepared by different methods, met the usual criteria of protein purity.

Botulinus toxin is 15,000 times as active on a weight basis as the most toxic drug known, aconitin, and a molecule of toxin is 200 million times as toxic as a molecule of the drug (5). The M.L.D. for a 20 gram mouse is 3×10^{-11} grams of crystalline toxin, and if man is as susceptible on a body weight basis as the mouse, 0.25 micrograms of the pure toxin would kill a 70 kilogram man (6). Finally, to express its activity in terms recently used by an official of an international health organization, seven ounces distributed properly would kill the entire population of the world (5).

The toxin molecule has a molecular weight of 900,000. This brings up the question: how does this toxin injure the tissues of higher animals? Chemical analysis has failed to answer this question. The toxin is made up of proteins composed of the same amino acids found in the normal tissue

proteins of the host itself. In the case of type A botulinus toxin, a complete amino acid analysis has revealed no unusual chemical groupings that might provide a clue as to why it is toxic.

The calculated elementary formula of the toxin is:

$C_{40,298}\ H_{62,679}\ N_{10,472}\ O_{12,634}\ P_{15\text{-}17}\ S_{123}$.

Its amino acid composition is represented by the expression:

$Glycine_{166}$, $Alanine_{394}$, $Valine_{406}$, $Leucine_{708}$, $Isoleucine_{820}$, $Proline_{203}$, $Phenylalanine_{64}$, Cystine SH_{20}, $(Cystine\ S\text{-})_{40}$, $Methionine_{64}$, $Tryptophane_{82}$, $Arginine_{239}$, $Histidine_{60}$, $Lysine_{477}$, $Asparagine_{1370}$, $Glucine_{953}$, $Serine_{374}$, $Threonine_{642}$, $Tyrosine_{672}$ (7).

Comparison of the effects of botulinus toxin and curare (8) has appeared in the literature for many years but Guyton and MacDonald (6) have recently presented work which indicates that its action is different from that of curare. Acetylcholine and nicotine injected intra-arterially still caused contraction of the muscle after botulism poisoning. With curare poisoning such is not the case. This indicates a fundamental difference between curare and botulinus toxin. Evidence is presented which indicates that the principal action of botulinus toxin is probably at the myoneural junction, though possibly in the terminal nerve fibrils.

About the treatment of botulism poisoning Guyton and MacDonald (6) said,

> "Treatment of botulinus poisoning consists of massive doses of antitoxin, the use of artificial respiration and in cases of severe poisoning, the administration of vasoconstrictor drugs. The fact that poisoning lasts for many months makes the results of such treatment discouraging. The use of artificial respiration for several months or longer is not practical, and if a patient is poisoned sufficiently to require vasoconstrictor drugs he will probably die anyway. The only real salvation seems to be the early use of antitoxin in doses greater than 100,000 units of multivalent serum. Though antitoxin has been shown to be of value for guinea pigs as long as two days after poisoning, it is still true that its effect decreases exponentially with time. One must remember that once toxin has reached the nerve ending and produced its damage this action is irreversible for many months."

Lamanna (9) observed that type A botulinus toxin will cause hemagglutination of a red cell suspension. After this work was published many believed that this method of determining toxin activity would replace the experimental animal. Since then Lamanna (10) has found a lack of identity between hemagglutination activity and toxicity of the toxin.

There have been previous reports in the literature of attempts to inactivate the toxin. Abrams (4) found that at room temperature the toxin was most stable between pH 1.0 and 6.0 with maximum stability between pH 4.0 - 5.0, while above pH 7.0 the toxin was rapidly destroyed. He found also that a temperature of 60 C. at pH 5.0 was

sufficient to destroy its activity. Their method was most peculiar in that they treated the toxin with formalin for one hour at room temperature and then refrigerated it for 18 hours before testing. Samples treated with hydrochloric acid were kept at room temperature for one hour and a half and then refrigerated for 18 hours. This may have been done because they were testing hemagglutinating activity of the toxin as well as toxicity. Jude et. al. (11) reported on their efforts to inactivate the type D botulinus toxin. Type D toxin was sensitive to potassium permanganate, to an organic form of iodine, and a quaternary ammonium derivative.

Bellinger et. al. (12) reported that after standing for 12 hours at 18 C. neither patulin, strepomycin, penicillin, hydroquinone, benzalacetonethiosemicarbazone, patulin thiosemicarbazone, 4-formylantipurine thiosemicarbazone, antihestimic drugs nor vitamin D2 had any effect on the botulinus toxin. The oxidizing agents potassium permanganate and a solution of elementary bromine in distilled water neutralized this toxin while quinone, quinhydrone, and hydrogen peroxide as well as aldehyde compounds failed to do so. Of the numerous dyes tested, only crystal violet detoxified botulinus toxin after one hours combination.

A recent communication by Littauer (13) indicates that five percent copper sulfate and silver ions were used with no adverse effects on the type A toxin. Potassium iodomercurate in concentrations of one and two-tenths percent caused inactivation.

III. MATERIALS AND METHODS

A. Medium and Organism

The work by Lewis and Hill (14) has shown that clarified corn steep liquor (two-tenths to four-tenths percent total solids), two percent powdered skim milk or one-half percent casein (technical grade), two-tenths to six-tenths percent commercial grade glucose (cerilose) at a pH of 6.8 to 7.6 gives a high yield of toxin. This medium is inoculated with two percent of an actively growing culture of the "Hall strain" of Clostridium botulinum, type A, and incubated at 35 C. for 48 to 72 hours.

B. Experimental Animals

The mouse was selected because of its sensitivity to the toxin. The mice used were from 16 to 24 grams for preliminary work and 18 to 22 grams for final determinations.

C. Dosage

Five-tenths of a ml. inoculated intraperitoneally was used because it is believed that this is the most sensitive method for determining toxicity.

D. Dilution of Toxin for Injection

A buffer made up of two-tenths percent gelatin phosphate solution adjusted to pH 6.5 was used in the early experiments. In the later experiments all dilutions were made with sterile distilled water because under the conditions of these experiments no appreciable loss in toxin titer was observed.

E. Toxicity

The toxicity was determined on a LD_{50} basis rather than the M.L.D. because it is statistically more valid (3). Eight animals were used at a given point but for preliminary work three animals were used. The animals were checked daily for five days.

F. Time of Contact of Agent with Toxin

All inactivating agents except the methylene blue chloride, ultra-violet light, and ion-exchange resins were allowed to remain in contact with the botulinus toxin for 30 minutes at room temperature (22 - 25 C.). Samples that were treated with chlorine were kept at room temperature for 30 minutes with enough chlorine to give a solution that had a no chlorine demand, then they were treated with the experimental dosage and allowed to stand for 30 minutes.

In all experiments except with the use of active carbon and ion-exchange resins the chemicals were added to 10 ml. of toxin-water solution.

To 100 ml. of toxin-water solution active carbon (activated charcoal) was added to give the desired concentrations. The solution was shaken in a dilution blank 25 times, then poured into centrifuge bottles and centrifuged at 100 x G for 30 minutes.

Methylene-blue chloride photodynamic effect was determined by exposing the toxin-dye solution in a Bioassay Petri dish to a 200 watt electric light bulb at a distance of 15 centimeters for 15 minutes.

The ultraviolet light (General Electric Germicidal Lamp - 15 watts) was placed two centimeters away from the toxin in a Bioassay Petri dish and was exposed for periods of one and ten minutes.

The column technique was used for most of the experiments with ion-exchange resins. Thirty grams of resin was placed in the column and then regenerated. The resin was then washed well with water to get rid of the excess regenerant. The toxin-water solution was then poured through the column very slowly.

In the batch technique 10 grams of resin was regenerated, then washed well with water, and all of the excess water poured off. The toxin-water solution

was added to the resin in a dilution blank and then shaken 25 times. The supernatant was withdrawn for testing after 15 minutes.

G. Controls

All chemical compounds were tested intraperitoneally the same as the toxin. Controls were allowed to stand for 30 minutes and the highest concentration used in the experiment was used as a control.

H. Chlorine Determinations

Concentrations of the chlorine solutions were determined by the amperometric method of titration (15). Chlorine demand of the toxin was found by the Ortho-Tolidine-Arsenite Test (16).

IV. RESULTS AND DISCUSSION

Preliminary Results

Table I

Effect of Chemical on 150 Mouse LD_{50}'s

Chemical	Conc. ppm	Deaths
Formaldehyde	100	3/3
Roccal	100	0/3
Chlorine	25	3/3
"	50	3/3
"	100	3/3
"	150	3/3
Potassium Permanganate	100	3/3 *
Colloidal Iodine	100	0/3

* Controls also died

Table II

Effect of Chemicals on 500 Mouse LD_{50}'s

Chemical	Conc. ppm	Deaths
Formaldehyde	100	3/3
Roccal	10	3/3
Chlorine	25	3/3
Potassium Permanganate	100	2/3
Colloidal Iodine	10	1/3

Table III

Effect of Chemicals on 30,000 Mouse LD_{50}'s

Chemical	Conc. ppm	Deaths
Formaldehyde	200	3/3
Roccal	200	3/3 *
Chlorine	50	3/3
"	100	2/3
"	200	0/3
Potassium Permanganate	200	0/3
Colloidal Iodine	50	3/3

* Controls also died

Table IV

Effect of Chemical on 240 Mouse LD_{50}'s

Chemical	Conc. ppm	Deaths
Beta Propiolactone	5	3/3
"	12.5	3/3
"	25	3/3
"	50	3/3

The first chemical given consideration was chlorine because it is being used in this country for treating many water supplies. Tables I, II, and III show that it would take 200 ppm to inactivate the toxin. This would be too much chlorine to use in water treatment.

Potassium permanganate was the next chemical considered because it has been used in some countries for water treatment. Tables I, II, and III show that 100 ppm had some activity and 200 ppm inactivated the toxin. Here again the amount of permanganate is too high for practical consideration.

Some of the other chemicals were of theoretical interest to give an indication of how certain chemical groups might act. Many of the compounds could not be used in treating water because of toxicity and the others could not be used until long term toxicities were determined.

Tables I, II, and III indicate that formaldehyde at 200 ppm did not inactivate botulinus toxin.

The results in Tables I, II, and III reveal that iodine at 10 ppm had some effect on 500 LD_{50}'s and 100 ppm inactivated 160 LD_{50}'s but 50 ppm did not inactivate 30,000 LD_{50}'s.

While Table I demonstrates that Roccal inactivated 160 LD_{50}'s it turned the toxin-water solution milky. Table II indicates that 10 ppm had no effect on 500 LD_{50}'s. Table III discloses that 200 ppm of Roccal was toxic for the mouse.

Table IV shows that beta propiolactone (50 ppm) had no apparent effect on 340 LD_{50}'s of toxin.

Table V

Effect of Hydrogen Ion Concentration on 5,012 Mouse LD_{50}'s

pH 8.40

Dilution of Sample	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	3/3	3/3	3/3	0/3

pH 10.10

Dilution of Sample	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	3/3	3/3	3/3	0/3

pH 12.55

Dilution of Sample	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	0/3	0/3	0/3	0/3

High pH had a definite effect on the toxin. Toxin was inactivated at pH 12.55. At pH 8.40 and pH 10.10 there was some inactivation.

Water plants that are softening their water with the lime-soda process would partially inactivate the toxin.

Table VI

Effect of 400 ppm of Beta-Propiolactone on 10,000 Mouse LD_{50}'s

Dilution of Sample	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	3/3	3/3	3/3	3/3

Beta-Propiolactone was of interest because it has been shown to be effective against bacteria and viruses in plasma and blood (17). It is an acylating agent possessing a low degree of toxicity (18). However, it had no activity against the toxin at the concentration used in these experiments.

Table VII

Photodynamic Effect of Methylene Blue Chloride on 10,000 Mouse LL_{50}'s

Methylene Blue Chloride 1:2,000

Dilution of Sample	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	3/3	3/3	3/3	0/3

Methylene Blue Chloride 1:100,000

Dilution of Sample	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	3/3	3/3	3/3	3/3

Methylene blue chloride was shown to be capable of inactivating tetanus toxin by an oxidation phenomena (19). It showed a slight effect against botulinus toxin at a concentration of 1:2,000 of the dye.

Table VIII

Effect of Active Carbon (Aqua Nuchar A) on 5,012 Mouse LD_{50}'s

70 ppm of Active Carbon

Dilution of Sample	10^{0}	10^{-1}	10^{-2}	10^{-3}
Deaths	3/3	3/3	3/3	3/3

140 ppm of Active Carbon

Dilution of Sample	10^{0}	10^{-1}	10^{-2}	10^{-3}
Deaths	3/3	3/3	3/3	3/3

Under the experimental conditions active carbon did not remove any of the botulinus toxin from water.

The use of active carbon was of particular interest because it is used in some water treatment plants to remove particulate matter and excess chlorine.

Table IX

Effect of Ultraviolet Light on 1,000 Mouse LD_{50}'s

One Minute Exposure at a Distance of Two Centimeters

Dilution of Sample	10^{0}	10^{-1}	10^{-2}	10^{-3}
Deaths	3/3	3/3	3/3	3/3

10 Minutes Exposure at a Distance of Two Centimeters

Dilution of Sample	10^{0}	10^{-1}	10^{-2}	10^{-3}
Deaths	3/3	3/3	3/3	3/3

Ultraviolet light was used because it was thought it might denature the protein but it had no apparent effect.

Table X

Effect of Passing 100 milliliters of 1,000 Mouse LD_{50}'s of Toxin Through 30 grams of Strong Base Exchange Resin-Hydroxy Form (Amberlite XE-98) in a Column.

Dilution of Sample	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	0/3	0/3	0/3	0/3	0/3

Table XI

Effect of Passing 100 milliliters of 10,000 Mouse LD_{50}'s of Toxin through 30 grams of Strong Base Exchange Resin-Hydroxy Form (Amberlite XE-98) in a Column.

Dilution of Sample	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Deaths	0/3	0/3	0/3	0/3	0/3

An anion exchange resin was reported by LoGrippo (20) to absorb the Lansing strain of the Poliomyelitis virus and Theiler virus (TO).

Final Results

Table XII

Effect of Passing 100 milliliters of 10,000 Mouse LD_{50}'s of Toxin Through 30 grams of Strong Base Exchange Resin-Hydroxy Form (Amberlite XE-98) in a Column.

Dilution of Sample	10^0	10^{-1}	10^{-2}
Deaths	0/8	0/8	0/8

Table XIII

Effect of Using the Batch Method of Ion Exchange on 100 milliliters of 10,000 Mouse LD_{50}'s with 10 grams of Strong Base Anion Exchange Resin-Hydroxy Form (Amberlite XE-98).

Dilution of Sample	10^0	10^{-1}	10^{-2}
Deaths	0/8	1/8*	0/8

* Not a typical botulism death

A strong base anion exchanger used in the hydroxy form proved to be successful in removing the toxin from water. Since strong base anion exchange resins are used in water conditioning for the removal of silica from water fed to high-pressure boilers (21) this should prove to be a practical method for removing type A botulinus toxin from a water supply.

V. SUMMARY

1. A study has been made of a number of chemicals in an attempt to inactivate botulinus toxin type A. None of the chemicals used were effective in low enough concentrations to be of practical value for use in treating a water supply because of toxicity. Chlorine was of primary interest because it is used by many water treatment plants.

2. Evidence is given which indicates that a strong base anion exchange resin removes type A botulinus toxin from water.

3. Further research should be done to determine if ion exchange resins could be used to concentrate the toxin and purify it. The concentration of the toxin would be helpful in detection in case of attack during wartime.

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