THE USE OF ION-EXCHANGE RESINS FOR THE PURIFICATION OF TYPE E BOTULINUS TOXIN

Thesis for the Degree of Ph. D.
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Alfred Mills Wallbank
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This is to certify that the

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The Use of Ion-Exchange Resins

for the Purification of Type E Botulinus Toxin

presented by

Alfred Mills Wallbank

has been accepted towards fulfillment
of the requirements for

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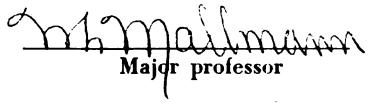

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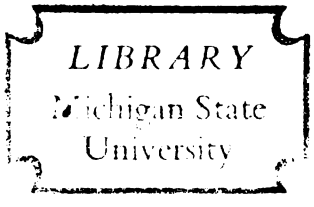

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By

Alfred Mills Wallbank

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Approved A. M. Wallbank

Pure type A botulinus toxin is the most active toxic substance known at the present time. One milligram will kill 1,200 tons of living matter. The possible use of the toxin in biological warfare, and interest in why it is more toxic than other compounds, led to this study of type E Clostridium botulinum toxin.

Both types A and B toxin have been purified. Type E botulinus toxin, which is the only other type toxic for humans, had not been purified at the time this study was undertaken, but while the work was in progress, it was purified by ethanol precipitation at Fort Detrick, Maryland by Gordon et al. (1).

This study was an attempt to purify type E toxin using ion-exchange resins. At the beginning of this work there was no method available to produce high concentrations of type E botulinus toxin. The study was started with type A botulinus toxin and was changed to type E toxin when a method was found to produce sufficient type E toxin.

A strong basic anion exchanger was used to absorb crude type A toxin but no method was found to elute the toxin from the exchanger. Then, type A toxin was adsorbed by a strong cation exchange resin and a toxic fraction was eluted with a phosphate buffer at pH 7.0.

Since the strong cation exchanger was successfully used in adsorption of the type A toxin, it was tried with the crude type E toxin with favorable results. Again, as with the type A toxin, a toxic fraction was eluted from the type E toxin with a phosphate buffer at pH 7.0. The eluate moved as one component in paper electrophoretic experiments but did not have the same mobility as any of the components of the crude toxin.

Using the Ouchterlony technique, the eluate did not give evidence of containing an antigenic component identical to any of those appearing in crude type E toxin, which would lead to the assumption that the eluate toxicity was caused by a molecule antigenically unrelated to the original fraction.

Type A toxin loses 50 to 75 per cent of its toxicity upon filtration through filters for bacteriological filtration. Type E toxin was not as labile, for filtration through a Selas 015 filter had no significant effect on the titer of crude type E toxin. Also storage in a freezer at -23 C did not reduce the titer of the toxin.

This study has presented no evidence that type E botulinus toxin can be purified by ion-exchange resins. While the use of ion-exchangers has not given the desired results in these studies, the writer believes that they should be given consideration in purification of metabolic products of microorganisms.

REFERENCE

1. Gordon, M.; Fiock, J. A.; Yarinsky, A.; and Duff, J. T. Purified Clostridium botulinum type E Toxin and Toxoid Bact. Proc. p. 93, 1956

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A THESIS

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TO BARB

VITA

Alfred Mills Wallbank

candidate for the degree of

Doctor of Philosophy

Final examination: November 30, 1956, 3:00 P.M., Room 300, Giltner Hall

Dissertation: The Use of Ion-Exchange Resins for the Purification of Type E Botulinus Toxin

Outline of Studies:

Major Subject: Microbiology
Minor Subject: Biochemistry

Biographical Items:

Born: May 13, 1925, Farmington, Michigan

Undergraduate Studies: Michigan State College, 1944-1948, B.S., 1948

Graduate Studies: Michigan State University, 1951-1956,
M.S., 1953
Major Professor - Dr. W. L. Mallmann
Thesis Title: Removal of Botulinus Toxin from Water.

Ph. D., 1956
Major Professor - Dr. W. L. Mallmann

Experience: Bacteriologist, Barry Laboratories, Detroit, Michigan, 1948-1950.
Bacteriologist, Henry Ford Hospital, Detroit, Michigan, Blood Sterilization Project, 1950-1951.
Graduate Teaching Assistant, 1953-1956.

Member: Society of American Bacteriologists,
The New York Academy of Sciences,
Society of Sigma Xi.

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I. INTRODUCTION

Botulinus toxin has been suggested as a lethal agent in biological warfare (1). Since 0.25 μg of the pure toxin would kill a 70 kilogram man (2) or seven ounces distributed properly would kill the entire population of the world (3) it would appear that botulinus toxin is highly suitable for warfare. Because of its possible use, an impetus to its study as to production, means of control, and the nature of the toxin itself, has occurred.

In a Masters Thesis (4) the writer was concerned with destruction or removal of botulinus toxin in municipal water supplies. He found that chlorine would inactivate type A botulinus toxin but only in high concentrations that could not be applied practically in water purification systems. An ion-exchange resin was tried and was found effective in removing the toxin.

Sober, Kegeles, and Gutter (5) have proposed that homogenity in ion-exchange chromatographic analysis be considered one of the required criteria of protein purity. Since type A botulinus toxin, which is considered to be a protein (6), was adsorbed by ion-exchange resins, elution should give a "pure" protein.

Both type A and B toxin have been purified. The only type of botulinus toxin left which is toxic to man was type E. Therefore a study using ion-exchange resins for purification of type E toxin was undertaken.

There are many other methods available today for purification of proteins, but these methods necessitate either elaborate equipment or long involved chemical procedures. A method which can be used for purification of proteins, requiring only a glass column and a few grams of ion-exchange resin, could be used in practically any laboratory.

II. REVIEW OF THE LITERATURE

A. Botulism

Botulism is an intoxication which was first described in 1820 by the German poet and medical writer Justinus Kerner (7). The study of botulism, particularly of the toxin responsible for its manifestation, has led to a wider basic understanding of bacterial toxins and their role in disease.

The term botulism (from Latin botulus, a sausage) was first applied in south Germany to the paralytic toxic syndrome resulting from the ingestion of spoiled sausage. In 1896 Van Ermengem (8) isolated from a ham a gram positive large rod which he called Bacillus botulinum, which was responsible for an outbreak of botulism in Belgium. Today, these organisms are placed in the genus Clostridium, comprising spore-bearing anaerobic bacilli with a species designation of Clostridium botulinum or Clostridium parabotulinum. Van Ermengem demonstrated, as Roux and Yersin had similarly shown for diphtheria, that botulism was caused by a toxin produced by the botulinum organism. Kempner (9) observed that antitoxin could be produced in experimental animals by immunization with the toxin produced in cultures of the botulinum bacillus. These two reports form the basis for the subsequent widespread research into the problems of botulism and the nature of the toxin.

Two groups of workers both working independently

at Fort Detrick during World War II succeeded in purifying and crystalizing type A botulinus toxin. Lamanna, Eklund, and McElroy (10) based their method upon the observations of Snipe and Sommer (11), and Sommer (12), that the toxin is acid precipitable from culture medium and can be eluted from the acid precipitate by buffer solutions at appropriate pH values. Abrams, Kegeles, and Hottle (13) used the usual methods of alcohol and salt fractionation of proteins. By both these methods of purification the protein met the usual criteria of protein purity.

This purified type A toxin is one of the most highly toxic compounds known to man. The purified 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 (3). The MLD for a 20 g mouse is 3×10^{-11} g of crystalline toxin.

The interesting question now arises; 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 protein 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 prove 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-) 40, Methionine 64, Tryptophane 82, Arginine 239, Histidine 60, Lysine 477, (Asparagine $-NH_2$) 1370, Glutamic acid 953, Serine 374, Threonine 642, Tyrosine 672 (6).

Comparison of the effects of botulinus toxin and curare has appeared in the literature for many years but Guyton and MacDonald (2) have recently presented data which indicate that its action is different from that of curare. Acetylcholine injected intra-arterially still caused contraction of the muscle after botulism poisoning. With curare poisoning, intravenous injection of acetycholine does not cause contraction of the muscle. 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 stated,

> "Treatment of botulinus poisoning consists of massive doses of antitoxin, the use of artificial respiration and in the 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 the toxin has reached the nerve ending and produced its damage this action is irreversible for many months."

B. Types of Clostridium Botulinum

Leuchs (14) found that certain cultures of the botulinum bacillus, isolated from various sources, produced a specific toxin neutralized only by its homologous antitoxin. In 1924 Bengston (15) distinguished the species of C. botulinum on the basis of their ovalytic (digestion of egg white) activity. Van Ermengem's isolate was called C. botulinum and was non-ovalytic. The American species was called C. parabotulinum and was ovalytic. Today emphasis in classifying organisms belonging to the botulinus group rests on the toxin-antitoxin neutralization specificity. Burke (16)classified 12 American strains on this basis as A or B.

On the basis of their specific toxins five types of C. botulinum have been recognized. They are designated as C. botulinum or parabotulinum, type A, B, Cα, Cβ, D, and E (17). Neutralization of toxin by the homologous antitoxin is fairly constant and few discrepancies have been

noted. Pfeenninger (18) reported in 1924 that antitoxin produced against the toxin of an Australian strain, resembling type C and whose toxin was neutralized by type C antitoxin, would neutralize the homologous toxin but not the toxins produced by other type C cultures. This interesting observation indicated that some variation in type specificity can take place.

C. Clostridium Botulinum Type E

Incidence

Type E was not recognized until 1936 when Gunnison, Cummings, and Meyer (19) reported on two cultures of C. botulinum sent to them by Dr. L. Bier of the Bacteriologic Institute at Dniepropetrowsk, Ukrania, U.S.S.R. Toxin-antitoxin neutralization tests in mice and guinea pigs revealed that antitoxins of types A, B, C, and D in doses adequate to protect against 250 to 170,000 MLD of homologous toxin failed to protect against two to five MLD of toxin produced by these cultures. Conversely, they found that antitoxin produced by this toxin failed to protect against two to three doses of types A, B, C, and D toxins. On the basis of these results they proposed the designation of C.botulinum type E for these cultures. They further pointed out that the designation of the C. parabotulinum equi of Theiler and Robinson as type E in Topley and Wilson, Second Edition, 1936, p. 688, was incorrect since the organism of

equine botulism belongs to type C. (It would seem that its neutralization by type C or E antitoxin is of more importance than its effect on the horse).

In 1937 Hazen (20) reported on a strain of C. botulinum isolated in December, 1934, from German canned sprats, which had been established as the cause of three cases of botulism. In vivo and in vitro attempts to neutralize the toxic filtrate obtained from this culture by monovalent botulinum antitoxic sera types A, B, and C resulted in failure. In 1938, Hazen (21) reported the isolation of C. botulinum from salmon, smoked and canned in Nova Scotia (later corrected by Dolman and Kerr (22) and established as Labrador), and implicated in another outbreak of botulism in New York State. With the cooperation of Dr. K. F. Meyer of the Hooper Foundation, San Francisco, she was able to identify her two cultures as belonging to type E.

Since then many other incidents involving type E have been reported; Geiger (23), 1941; Dolman and Kerr (22), 1947; Dolman, et al. (24), 1950; Prevot and Huet (25), 1951; Meyer and Eddie (26), 1951; Sakaguchi, et al. (27), 1954. In Table I are summarized the reports appearing in the literature on the various isolations of C. botulinum type E that have been identified.

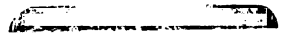


TABLE I

Reported Isolations of *Clostridium botulinum* Type E from Food:

Year Reported	Reported By	Source of Isolation	Country	Designation of Strain
1936	Gunnison, Cummings, & Meyer(19)	Russian Sturgeon	Soviet Ukraine	Russian 151 Russian 30-17
1937	Hazen (20)	German canned sprats	Westchester County, N.Y.	Sprat No. 35396 (A.T.C.C.9565)
1938	Hazen (21)	Labrador smoked salmon	Coopers-town, N.Y.	Salmon No. 36208 (A.T.C.C.9564)
1941	Geiger(23)	Yugoslav mushrooms canned in California	San Francisco, Calif.	Mushroom
1947	Dolman & Kerr (22)	Home canned salmon	Nanaimo, B.C., Canada	Nanaimo
1950	Dolman, Chang, Kerr & Shearer(24)	Home pickled herring	Vancouver, B.C., Canada	VH
1951	Prevot & Huet (25)	Fresh perch	France	French
1951	Meyer & Eddie (26)	Uncooked whale flappers	Point Hope, Alaska	No designation to date
1951	Sakaguchi *et al.* (27)	Herring-Izushi	Japan	No designation to date
1952	"	Flatfish-Izushi	"	"
1952	"	Flatfish & Dace-Izushi	"	"
1953	"	Flatfish-Izushi	"	"
1953	"	Gilthead-Izushi	"	Tenno

Toxigenic Properties

Type E toxin affects laboratory animals and man in much the same way as do the toxins of the other human botulinum types (A and B) as far as can be judged by the symptoms that develop. The symptoms are disturbance of vision, muscular paralysis and respiratory failure.

Most investigators have found that the toxin production in culture media with type E strains is quite variable. The Canadian workers have obtained high toxin yields with the VH (Vancouver-Herring) strain of Dr. Dolman (22).

By conventional culture methods type E toxin yields have been lower than the other two types (A and B) which cause human botulism. Toxigenic type A cultures (particularily the Hall strain) regularly produce titers of 500,000 to 1,000,000 MLD per ml of culture for the white mouse. Rice, Smith, Pallister, and Reed (28) report titers of 600 to 1,400 MLD per ml with type B for the mouse, but point out that they found the mouse much more resistant to type B toxin than the guinea pig. Nigg, et al. (29) obtained titers of 8,000 mouse MLD per ml with their type B culture.

Early work with the type E culture by Dolman, et al. (22) yielded 4,000 MLD per ml with the VH strain. Barron (30, 31) has adapted the method of Sterne and Wentzel (32) who described a double surface dialyzing membrane arranged by intussuscepting ("invaginating") cellophane bags for the

large scale production of types C and D botulinum toxin and toxoid.

The "invaginated" cellophane bag is filled with water or saline (0.85%) and suspended in the culture medium and the entire unit sterilized. The saline is inoculated with the organism and the culture allowed to incubate. Dialysis of the medium, according to Sterne and Wentzel, is an essential part of the method. As the culture grows waste products presumably dialyze into the medium surrounding the bag, while fresh nutrients continue to dialyze into the bag. Since botulinus toxin is non-dialyzable the elaborated toxin accumulates inside the bag. The maximum titer is reached, when incubated at 30 C, in 10 days. The toxin is harvested by withdrawing the contents of the bag. Sterne and Wentzel referred to the toxins produced in the cellophane bag as "dialysate" toxins.

After this work had been in progress for some time Gordon, et al. (33) at Fort Detrick, Maryland, purified type E toxin by a procedure involving precipitation of toxin by ethanol in the cold, extraction of toxin from the precipitate with calcium chloride solution and reprecipitation twice with ethanol in the cold. The product obtained contained 9×10^4 $LD_{50/mg}$ N. Duff and co-workers (34) also reported a study on the activation of type E toxin by trypsin. Their investigations showed that the toxicity of cultures of C. botulinum were increased from 12 to 47 fold by treatment

for one hour with one per cent trypsin (Difco 1:250) at pH 6.0 and 37 C.

A group of Japanese workers have isolated an anaerobic organism which appears to belong to the genus Clostridium which increases type E toxin production in a mixed culture. They have called this organism strain No. 13. This strain itself was proved to be non-toxigenic for mice. Further work has shown that a sterile culture filtrate of strain No. 13 will give higher titered toxin from type E organisms and is likely to be an enzyme in regard to its action and protein like properties (27, 35, 36, 37).

D. Use of Ion-Exchangers to Purify Viruses

Muller (38) was the first to employ ion-exchange resins to aid in the purification of viruses. He used a cation exchange resin (XE-64) to remove inert nitrogenous material from suspensions of chick embryos and mouse brains infected with neurotropic viruses.

Lo Grippo (39) used XE-67, an anionic exchanger to adsorb the Lansing strain of poliomyelitis and Theiler virus to the resin along with nitrogenous material. The virus was then selectively eluted from the resin.

In his second paper Muller (40) adsorbed the PR8 strain of influenza virus with a cation exchanger (XE-64) and then eluted the virus from the resin with sodium chloride solutions.

Takemoto (41) indicates that a cation exchange resin, Nalcite HCR-X12 (Dowex 50), can be used to adsorb type A influenza virus from human throat washings. The virus can be isolated more frequently with the use of resin eluates than without the exchanger.

III. MATERIALS AND METHODS

A. Media

Barron's broth (31) was used for toxin production in this study. The medium is composed of:

Heart-infusion broth (Difco)	25 g
Tryptone (Difco)	10 g
Glucose (Reagent)	10 g
Calcium carbonate (Reagent)	5 g
Distilled water	1 L

pH 7.4 - 7.6

The pH was adjusted with N/10 sodium hydroxide. The medium was made up without glucose and autoclaved at 121 C for 75 minutes because of the large volume of medium. The glucose was added aseptically after being autoclaved at 116 C for 20 minutes.

Stock cultures were kept in cooked meat medium (Difco) and stored in the refrigerator. As cultures were needed the organism was transferred to fluid thioglycollate medium (Difco) and incubated for 24 hours to obtain an actively growing culture.

B. Organism

The VH strain of C. botulinum, type E, isolated from herring in Vancouver, Canada, by Dolman was used during this entire study. The VH strain was obtained from

Dr. Jack Konwalchuk of the Defense Research Board, Kingston, Ontario, Canada. All cultures were incubated at 30 C to obtain maximum toxin production.

C. Toxin Production

A nine liter Pyrex carboy was used for a medium container. (See Figure I).

The organisms were added to a cellophane bag suspended in six liters of Barron's broth. Using this method the high molecular weight portion of the medium was kept out of the toxin.

The cellophane used was 8.5 cm wide seamless cellulose tubing obtained from Visking Corporation, Chicago, Illinois. In order to "intussuscept" the tubing it was found that a thorough soaking of the cellophane in water greatly facilitated this procedure. The tubing, once attached to the unit, was kept moist since drying of the cellophane presented the possiblity of cracking and also made it difficult to handle. Care was taken to avoid damaging the cellophane in arranging the set up.

Five ml of an actively growing culture taken from fluid thioglycollate medium were added to 200 ml of Barron's broth in an eight ounce prescription bottle. After 48 hours incubation the supernatant was decanted to avoid the calcium carbonate on the bottom of the bottle. The bacteria

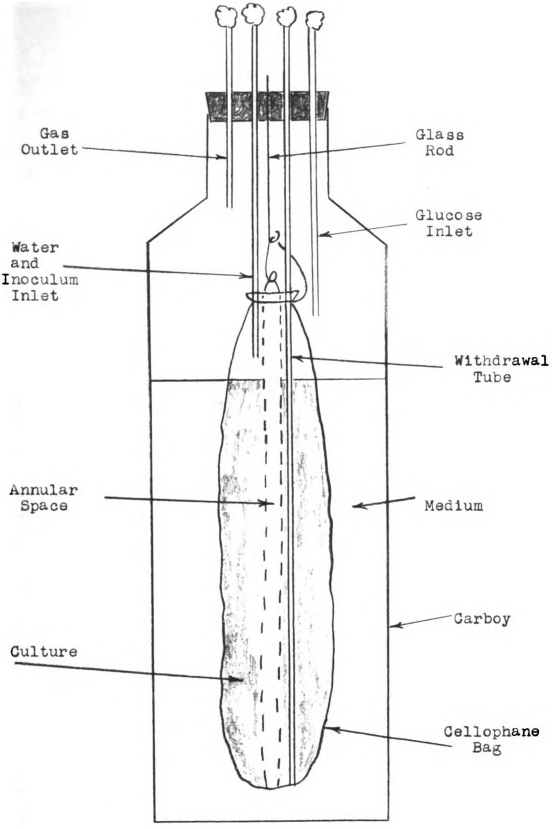


FIGURE I

"Intussuscepted" Cellophane Bag Unit

were then spun down in an International Refrigerated Centrifuge PR-1 (Angle Head) at 3,000 RPM for 30 minutes.

The organisms collected from one prescription bottle were resuspended in 350 ml of water and added to the "intussuscepted" cellophane bag in a carboy. Then, these large containers of broth were incubated at 30 C for 10 days. The contents of the bag were then aspirated into a flask. The toxin plus organisms were centrifuged in an International Refrigerated Centrifuge PR-1 (Angle Head) at 3,000 RPM for 30 minutes. The supernatant was poured off and filtered through an 015 Selas filter. The toxin was immediately put in screw capped test tubes and either frozen or refrigerated. A sterility test was made by adding two ml of toxin to both fluid thioglycollate medium and cooked meat medium. These tubes were incubated at 37 C for seven days.

D. Experimental Animals

The mouse was used for assay of the toxin because of its sensitivity to type A and type E toxin. Carworth Farms strain CF #1 was used for this entire study because of their uniform response in toxicity studies.

The mice were fed Rockland Mouse Diet in pellet form. The mice were housed in cages made of either stainless steel or Monel metal measuring 21 x 33 x 16 cm or 32 x 40 x 16 cm. The cages had suspended wire-mesh (0.8 cm mesh) food receptacles built into the center of the cover.

The water bottles were made available to the animals by inserting the glass tubes through the covers. Shavings were used for bedding and were changed once a week or more frequently if necessary. The room temperature was approximately 21 C, except during the summer months, when it was difficult to maintain this temperature without air-conditioning.

After discarding rough-furred or unhealthy looking mice, the remaining animals were separated into weight groups. Animals were selected at random from the 16 to 24 g group for preliminary work, and from the 18 to 22 g group for more critical experiments.

E. Dosage and Toxicity

The dosage of diluted toxin used was 0.5 ml inoculated intraperitoneally in the following manner: the mouse was picked up by the tail in the left hand, held by the fur on the back of the neck with the abdomen facing upward, and the needle was introduced at approximately a 45 degree angle into the abdominal cavity.

Preliminary toxicities were determined in terms of the minimum lethal dose (MLD). Using three mice per dilution, the highest dilution killing two or more mice was accepted as the MLD.

The lethal dose for 50 per cent of the mice (LD_{50}) was obtained by using eight mice per dilution and calculations made by the method of Reed and Muench (42).

The animals were checked daily for five days at the beginning of the work. After some time it was noted that only two deaths had occurred after three days; so to facilitate the work animals were checked for three days. Observation revealed that death was usually preceded by labored breathing and what appeared to be muscular paralysis.

F. Dilution of Toxin for Titration

Serial, tenfold dilutions of the toxin being titrated were made in a gelatin-phosphate buffer (43) consisting of 0.2 per cent gelatin in a one per cent phosphate buffer, pH 6.9, autoclaved at 121 C for 15 minutes. A different sterile pipette was used for each successive dilution.

G. Ion-Exchange Columns

The Pyrex glass columns used in these studies were 44 cm long x 2.5 cm in diameter. The bottom portion of the column had a removable sintered glass filter that would retain the resin, yet allow flow of a liquid. The columns were always cleaned in dichromate cleaning solution and were well rinsed before using.

H. Conditioning the Ion-Exchange Resin

The ion-exchange resin was added to the column in a slurry and allowed to settle out.

The new resins, as received from the manufacturers,

contained a small amount of fines, (extremely small particles of the resins) which had to be removed to prevent excessive resistance to downflow operations. Removal of the fines was accomplished by backwashing, which is the process of allowing enough water to flow up through the column to expand the resin bed 100 per cent. Backwash was continued until the fines had been washed from the bed.

To obtain the hydrogen cycle, the cation exchange resin was treated alternately with 500 ml of 1N sodium hydroxide and 500 ml of 1N hydrochloric acid. This procedure was repeated three times. The exchanger was washed to neutrality with distilled water after each reagent.

The sodium cycle with cation exchangers was obtained by treating the resin alternately with 500 ml of 1N hydrochloric acid and 500 ml 1N sodium hydroxide. The resin was then rinsed with distilled water until the effluent was at neutrality.

The Amberlite IRC-50 (Rohm and Haas) was treated with 500 ml of 2N sodium hydroxide followed by water, 2N hydrochloric acid, and washed with distilled water. Since the IRC-50 was to be used in the (NH_4^+) cycle, the resin was treated with ammonium hydroxide and finally washed with distilled water until the pH was approximately 7.0.

The anion exchange resins were treated as follows: 1N hydrochloric acid and then 1N sodium hydroxide to establish the exchanger in the hydroxide cycle; and with 1N

sodium hydroxide and 1N hydrochloric acid to maintain the resin in the chloride cycle. The exchangers were washed to neutrality with distilled water after each reagent.

I. Paper Electrophoresis

The power source was made according to the specifications of Kunkel (44). Whatmann filter paper No. 1, cut into 2 x 18 inch strips or 4 x 18 inch strips, was used for all experiments. The paper was rinsed with water several times and dried before use.

Both the crude type E toxin and eluate were dialyzed against the buffer used in the experiments. The buffer was changed five times over a period of 24 hours. The dialysis was done at 4 C.

Barbital buffers (pH 8.6) at an ionic strength of 0.05 for the five hour runs, and an ionic strength of 0.075 for the 18 hour runs, were used in this study (45).

The paper strips were immersed in buffer solution and blotted until almost dry. Strips were then placed on a siliconized glass plate (10 x 16 inch). The eluate (0.005 ml) was placed, by means of a pipette, in the exact center of one strip, and this procedure repeated with the toxin on another strip. A second siliconized glass plate of the same size was placed on top of the plate with the paper strips, and the two plates were secured by clamps.

The electrophoretic runs were made at room tem-

perature (23 to 25 C) at seven milliamperes for five hours or eight milliamperes for 18 hours. The paper strips, after each electrophoretic experiment, were dried at 37 C and then sprayed with a 0.5 per cent ninhydrin solution. Rubber gloves were used at all times for handling the paper.

J. Ouchterlony Technique

This technique is used for the identification of molecules (especially protein molecules) which are generally characterized better by immunologic specificity than are molecules having the same specificity by other tests.

The test consists of adding antigen (AG) and antibody (AB) to suitably spaced reservoirs in an agar plate. The AG and AB diffuse through the agar and when the concentrations of the two reactants are in equivalence a line of precipitation appears. These specific lines appear as straight lines or with only a slight arc. The non-specific lines appear as an arc around the wells (at least that has been the experience in these studies).

The agar used in this work consisted of the following: agar (Difco), 20.0 g; sodium chloride (Reagent), 8.5 g; methyl orange, 0.02 g; merthiolate, 0.1 g; and distilled water, 1 L; adjusted to pH 7.3 - 7.5.

Forty ml of the melted agar was added to each of three plates. After the agar had solidified, a No. 5 sterile cork borer was used to cut three cups out of the agar in

each plate, thus forming wells. Two-tenths ml of melted agar was pipetted into the bottom of the wells to form a seal. The outside edges of the three wells were 18 mm apart and formed an equilateral triangle.

In these tests, 0.2 ml of the following reagents were added once a day for seven days to their respective wells: in the first plate - toxin, normal serum, and antiserum; the second plate - eluate, normal serum, and antiserum; the third plate - toxin, eluate, and antiserum. After each of these additions the plates were returned to the 37 C incubator. After the last addition on the seventh day, the plates were incubated for 24 hours, and then stored at 4 C.

K. Production of the Antisera

Three to four kilogram rabbits were used for production of antisera.

The rabbits were given repeated intravenous injections of type E botulinus toxoid and then toxin. The toxoid was made by adding 0.5 per cent formalin to 2,000 mouse $LD_{50/ml}$ of type E toxin and incubating the mixture at 37 C for 10 days. During the incubation period the mixture was gently rotated daily to insure proper mixing. Type E toxin was diluted to 200 and 2,000 mouse $LD_{50/ml}$. The rabbit was first given 0.4, 0.8, 1.6, 3.5, and 5.0 ml of the toxoid and then 0.1, 0.2, 0.5, and 2.0 ml of the

200 LD_{50}/ml toxin at three day intervals. Fifty ml of blood were bled from the heart 10 days after the last inoculation. The serum from this blood was used for preliminary studies. Three weeks after the last inoculation the rabbit was given 0.5 and 1.0 ml of the 2,000 LD_{50}/ml toxin at three day intervals. After 10 days 50 ml of blood were again bled from the heart. The serum from this blood was used for the more critical studies.

The vessel containing the blood was allowed to stand undisturbed at room temperature until the blood was clotted and then placed in a 37 C incubator for two to four hours. The blood was then separated from the walls of the vessel, and placed at 4 C for 24 hours. The serum was removed and added to sterile screw capped test tubes. One ml of the serum was added to fluid thioglycollate medium for a sterility test.

L. Different Methods Employed for Type A Toxin

The work by Lewis and Hill (46) has shown that clarified corn steep liquor, 4 g total solids; powdered milk, 20.0 g; commercial grade glucose (cerelose), 6.0 g; and tap water, 1 L; adjusted to pH 7.4 - 7.6, dispensed into eight ounce prescription bottles, and sterilized in the autoclave for 20 minutes at 121 C, gives high yields of toxin. In this study this medium was inoculated with two per cent of an actively growing culture of the "Hall strain" of *Clos-*

tridium botulinum, Type A, from fluid thioglycollate medium (Difco) and incubated at 35 C for 48 to 72 hours.

After incubation the culture was centrifuged in an International Refrigerated Centrifuge PR-1 (Angle Head) for 45 minutes at 3,000 RPM. The supernatant was the crude toxin and it was not filtered.

M. Column Method for Adsorption and Elution of Toxin

The water level was brought to one inch above the conditioned resin in the column and then the toxin was allowed to flow through the column at the rate of 0.5 ml per minute. After the solution of toxin had passed through the resin, the solution used for elution was then passed through at the same rate. Both the toxin and eluate were collected in test tubes and immediately stored at 4 C until they were assayed. A portion of the original toxin solution was also stored at 4 C to obtain the titer of the toxin added to the column.

N. Ion-Exchange Resins

A solid exchanger must have three characteristics. First, it must contain ions of its own. Second, it must be insoluble in water under all conditions. Third, there must be enough space between its molecules so that other ions can move freely in and out of the solid. Low crosslinked resins have less selectivity for specific ions and are capable of

exchanging ions of high molecular weights.

An exchanger molecule is a long chain polymer. It may have either a negative or a positive charge. To neutralize this charge, smaller ions of opposite charge are present. It is these small ions, that are not held by bonds to the rest of the molecule, that exchange with the ions in solution. The chemical structure of two typical ion-exchangers is shown in Figures II and III.

Figure II shows a crosslinked styrene polymer that has been sulfonated which leads to a polystyrene sulfonic acid in which every benzene ring in the polymer contains one sulfonic acid group. The resulting resin is a strong acid and many commercial cation exchange resins have this general composition.

Figure III shows crosslinked polystyrene that has been chloromethylated and then reacted with tertiary amines to give a "strongly" basic anion exchanger.

The flow of inorganic salt solutions through a resin bed containing cationic, anionic, and both a cationic and anionic resin is shown in Figure IV. This will give some idea how ion-exchangers work in a simple system.

Cohn has described in simple terms the principles involved in the ion-exchange system:

> "The most useful method for the separation of components in biochemical mixtures seems to be that of "elution analysis". This is a two step process, wherein the

mixture is first adsorbed at the top of a column, previously prepared in a given form, and then eluted in such a manner as to bring each substance to the bottom of the column as a separate and distinct "peak" without significant change in the form of the exchanger. In general, the adsorption step is carried out under conditions where the affinities of the ions in question for the exchanger are maximal, or at least greater than during the elution sequence. Elution utilizes conditions which decrease the affinities, releasing the ions from the exchanger. In so far as the adsorption is due to ionic forces, elution conditions fall into two main groups, (1) simple increase in the concentration of the competing ion to the point where it displaces the adsorbed ions by mass action (ionic-strength adjustment) and, (2) change (usually decrease) in the charge of the adsorbed ion by pH change, complex formation, the use of a nonpolar solvent, etc., (charge adjustment).

The method of charge adjustment is obviously applicable only to ions with variable charge, a not unusual situation in biochemistry. Amino and carboxyl groups are readily ionized or deionized, phosphate esters become singly or doubly ionized, sugars form borate complexes, etc., at pH values that are not difficult to attain in the laboratory and usually are not destructive to the material under investigation." (47)

FIGURE II

CATION EXCHANGER

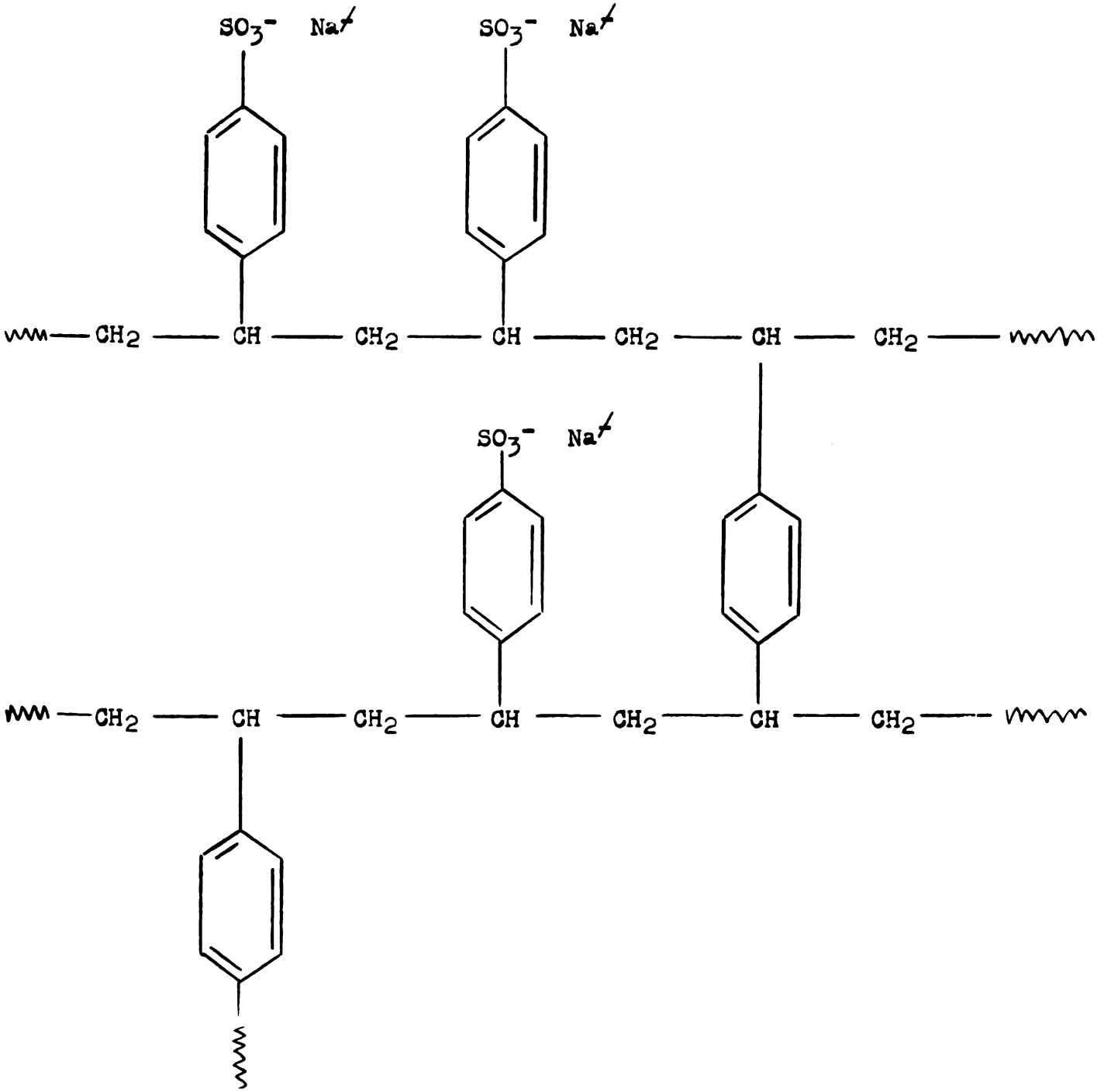


FIGURE III

ANION EXCHANGER

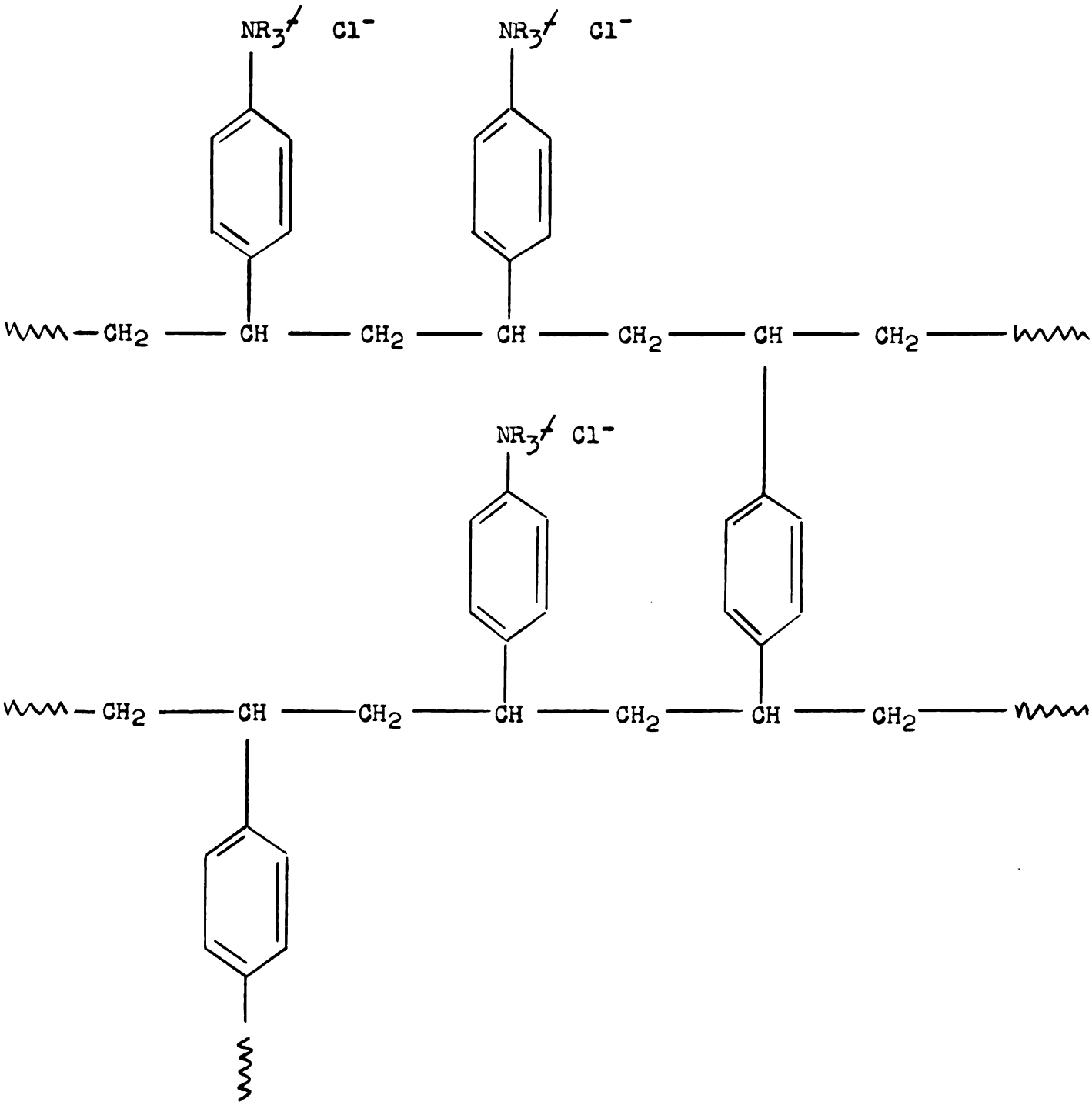
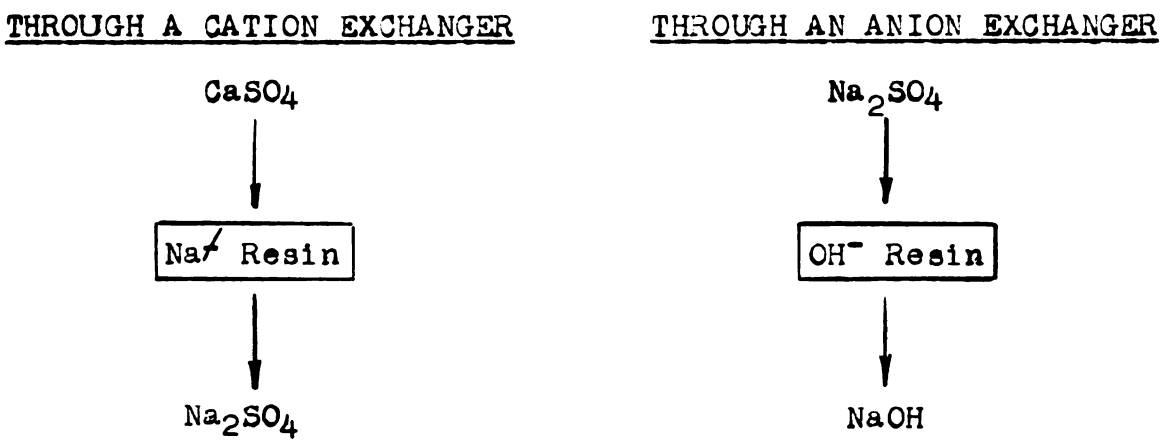
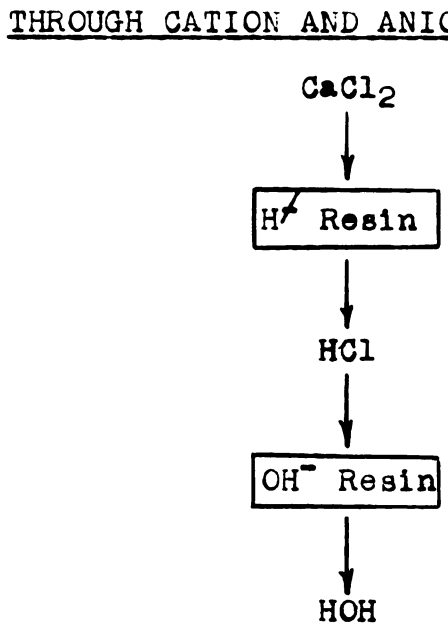


FIGURE IV

THE FLOW OF INORGANIC SALTS THROUGH ION-EXCHANGE COLUMNS





IV. RESULTS

At the beginning of this work there was no method available to produce high concentrations of type E botulinus toxin. This study was started with type A botulinus toxin but was changed to type E toxin when Barron and Reed (31) published their study on the production of type E toxin.

A. Type A Botulinus Toxin

It has been established that type A botulinus toxin can be adsorbed in a column containing Amberlite XE-98 (OH^-), a strong anion exchange resin (4).

In this study many buffers with different pH values and several chemicals of various concentrations were employed and found to be ineffective in eluting type A toxin from Amberlite XE-98 (OH^-). These different buffers and chemicals are listed in Table II.

It should be noted that 1.0N sodium hydroxide will inactivate type A toxin in 30 minutes, but 0.1N sodium hydroxide has very little effect in the same length of time (4). Therefore, the eluate was collected in beakers set up with a magnetic stirring apparatus, and a Beckman H-2 pH Meter. The pH of the eluate was adjusted to keep it in the range of pH 5.5 to 7.0.

As indicated in Table III many ion-exchange resins would not, at least in the cycle in these experiments, ad-

sorb type A toxin from solution. The anionic exchangers XE-67 (Cl^-), XE-98 (Cl^-) and IR-4B (OH^-) did not adsorb any toxin at all. It should be noted that XE-98 operating in the hydroxide cycle will adsorb toxin from solution. The cationic exchangers XE-66 (Na^+), 50-X2 (Na^+), 50-X8 (H^+), and IRC-50 (NH_4^+) also do not remove toxin from solution. Later it will be shown that both 50-X2 (H^+) and 50-X8 (H^+) will adsorb toxin under the proper conditions.

After unsuccessful attempts in using a cationic exchanger for the adsorption of type A toxin, the pH of the toxin solution was lowered below the iso-electric point (pH 5.6). This gave the protein a positive charge favoring the replacement of the hydrogen ion of the cationic resin with protein. This experiment was tried with type A toxin buffered to a pH of 4.2. A toxin solution (50 ml) containing 6,200 LD_{50}/ml was adsorbed by Dowex 50-X8 (H^+). The type A toxin was eluted from the column with a phosphate buffer at pH 7.0. (See Table IV).

TABLE II

Unsuccessful Methods of Eluting Type A Toxin From Amberlite XE-98 (OH^-)

(Resin - 13.5 x 2.5 cm in a column)

Experiment No.	pH of Toxin Before Adsorption	Chemicals for Elution and Fractions Collected
1	6.7	50 ml - 0.1N Sodium Chloride
2	7.2	100 ml - 10% Na_2HPO_4
3	5.5	10 ml - 0.1M Sodium Acetate plus 0.1M Acetic acid " - pH 4.1 Acetate buffer* 90 ml - pH 4.1 " "
4	6.7	10 ml - pH 4.6 Acetate buffer* " - pH 4.1 " " " - 1% Acetic acid " - 10% " " " - 1% Sodium Chloride " - 10% " "
5	6.9	50 ml - 0.001N Sodium Sulfate " - 0.01N " " " - 0.1N " " " - 1.0N " "
6	6.7	50 ml - 0.1N Sodium Hydroxide
7	7.4	100 ml - 0.1N Sodium Hydroxide " - " " " " - " " "

TABLE II (CONT.)

Experiment No.	pH of Toxin Before Adsorption	Chemicals for Elution and Fractions Collected
8	6.6	100 ml - 0.5N Sodium Hydroxide
		" - " " "
		" - " " "
		" - " " "
		" - " " "
		" - 1.0N " "
		" - " " "
		200 ml - Distilled Water
		" - " "
		" - " "
		" - " "
		" - " "
		100 ml - 0.5N Hydrochloric Acid
		" - " " "
		" - " " "
		" - " " "
		" - " " "
		500 ml - Distilled Water

(A flow rate of 10 ml per minute was used for this experiment)

*Buffers: See Appendix (#3 and #5)

TABLE III

Resins (In The Indicated Cycle) That Did Not Adsorb Type A Toxin From Solution

(Resin - 13.5 x 2.5 cm in a column)

Resin	Type	Cycle	pH of Toxin
Amberlite XE-67	Anionic	Cl^-	6.8
Amberlite XE-66	Cationic	Na^+	7.4
Amberlite XE-67	Anionic	Cl^-	6.7
Dowex 50-X2	Cationic	Na^+	6.7
Dowex 50-X2	Cationic	Na^+	7.1
Amberlite XE-98	Anionic	Cl^-	6.7
Dowex 50-X8	Cationic	H^+	7.4
Dowex 50-X8	Cationic	H^+	7.2
Amberlite IRC-50	Cationic	NH_4^+	7.5
Amberlite IRC-50	Cationic	NH_4^+	4.1
Amberlite IR-4B	Anionic	OH^-	6.7

TABLE IV

EXPERIMENT #9

Elution of Type A Botulinus Toxin from Dowex 50-X8 (H^+) (13.5 x 2.5 cm) with Phosphate Buffer, pH 7.0

(50 ml of Type A Toxin buffered to pH 4.2 Containing 6,200 LD_{50}/ml were added to the column)

10 ml Fractions	Eluate diluted 1-10 (No. of mice killed)
1	0/3
2	0/3
3	2/3
4	3/3
5	3/3
6	2/2
7	3/3
8	3/3
9	3/3
10	1/3
11	1/3
12	1/3
13	0/3
14	0/3
15	0/3
↓	↓
50	0/3

Buffers: See Appendix (#1 and #4)

B. Type E Botulinus Toxin

In view of the fact that Rice et al. (28) noted that bacteriological filtration removes from 50 to 75 per cent of the toxicity of type A botulinus toxin, a study was made of the effect of both filtration and storage in a freezer on type E toxin.

Neither filtration through a Selas 015 filter nor storage in a freezer for nine days at -23 C had any significant effect on type E botulinus toxin. (See Table V).

The next experiment was a successful attempt to adsorb type E toxin (50 ml), buffered to a pH of 4.2, containing 200 MLD/ml, with Dowex 50-X8 (H^+).

An experiment was arranged to ascertain if the type E toxin could be eluted with a phosphate buffer at pH 7.0. The type E toxin (50 ml), buffered at pH 4.2, containing 5,400 $LD_{50/ml}$, was added to the column. The toxin or some toxic component was eluted. (See Table VI).

The toxic portions of the eluate, which were pooled after preliminary titration, contained 2,000 $LD_{50/ml}$. The eluate gave a positive Biuret test and a negative Molisch test.

The results which are summarized in Table VII demonstrate again that a toxic fraction was eluted from Dowex 50-X8 (H^+). Type E toxin (50 ml), buffered at pH 4.2, containing 44,000 $LD_{50/ml}$, was added to the column, and phosphate buffer (pH 7.0) was used for elution. After preliminary titration the eluate was pooled. The eluate titer was 4,400 $LD_{50/ml}$.

TABLE V

The Effect of Filtration and Storage in a Freezer on Type E Botulinus Toxin

Control Titer	Filtered Toxin*	Stored in a Freezer**
26000 LD_{50}/ml	23000 LD_{50}/ml	32000 LD_{50}/ml

* 015 Selas Filter

** Nine days at -23 C

TABLE VI

EXPERIMENT #10

Elution of Type E Botulinus Toxin from Dowex 50-X8 (H^+) (13.5 x 2.5 cm) with pH 7.0 Phosphate Buffer

(50 ml of Type E Toxin buffered to pH 4.2 Containing 5,400 LD_{50}/ml were added to the column)

10 ml Fractions	Eluate Diluted 1-10 (No. of Mice Killed)	Titer in MLD	Titer of Pooled Toxin in LD_{50}/ml
1	0/3		
2	0/3		
3	0/3		
4	0/3		
5	0/3		
6	2/3	0	
7	3/3	2000	
8	3/3	2000	
9	3/3	20	2000
10	3/3	20	
11	3/3	0	
12	2/3	0	
13	2/3	0	
14	0/3		
15	0/3		
16	0/3		
17	0/3		
18	0/3		
19	0/3		
20	0/3		

Buffers: See Appendix (#2 and #4)

Flow rate - 10 ml/30 minutes

TABLE VII

EXPERIMENT #11

Elution of Type E Botulinus Toxin from Dowex 50-X2 (H^+) (13.5 x 2.5 cm) with pH 7.0 Phosphate Buffer

(50 ml of Type E Toxin Buffered to pH 4.2 containing 44,000 LD_{50}/ml were added to the column)

20 ml Fractions	Eluate Diluted 1-10 (No. of Mice Killed)	Titer in MLD	Titer of Pooled Toxin in LD_{50}/ml
1	0/3	0	
2	3/3	20	
3	3/3	20000	
4	3/3	2000	4400
5	3/3	2000	
6	2/3	200	
7	1/3	0	
8	2/3	20	
9	1/3	20	
10	0/3	0	
11	0/3	0	
12	0/3	0	
13	2/3	20	
14	0/3	0	
15	0/3	0	
16	0/3		
17	0/3		
18	0/3		
19	0/3		
20	0/3		

Buffers: See Appendix (#2 and #4)

Flow Rate - 10 ml/30 minutes

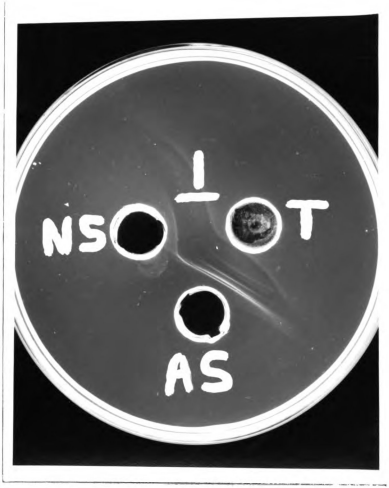
The Ouchterlony test was chosen as the most critical method to ascertain if the eluate contained toxin or if the eluate contained any fraction that was identical to one of the components of the toxin. For this test, an antiserum was prepared against the toxin and the antigens (eluate and toxin) were placed in the wells of an agar plate to react under standard conditions.

In figures V, VI, and VII are photographs of agar plates showing the results of the Ouchterlony test. In plate 1 (Figure V), which contained type E toxin (T), normal serum (NS), and anti-type E botulinus serum (AS), six lines of precipitation appeared, at 37 C, between the wells containing T and AS, revealing at least six antigenic components in the toxin. A line of precipitation appeared between T and NS when the plates were placed at 4 C (this was noticed after 10 day storage). The continuation of this line of precipitation between T and AS into T and NS indicates that toxin reacts with a component common to both AS and NS. In plate 2 (Figure VI) containing eluate (E), NS and AS, there were no specific lines of precipitation at either 37 or 4 C. Plate 3 (Figure VII), containing T, E, and AS had three lines between the T and AS wells, which signifies at least three antigenic components in the T. There were no lines of precipitation between E and AS, indicating the absence of any antigenic components common to the toxin. Plate 3, when placed at 4 C did not give any more lines of precipi-

FIGURE V

Ouchterlony Test

Plate 1



NS - Normal Serum

T - Type E Toxin

AS - Anti-Type E
Botulinus Serum

FIGURE VI

Ouchterlony Test

Plate 2

NS - Normal Serum

E - Eluate

AS - Anti-Type E
Botulinus Serum

FIGURE VII

Ouchterlony Test

Plate 3

T - Type E Botulinus Toxin

E - Eluate

AS - Anti-Type E
Botulinus Serum

tation. The toxin was the same material used in experiment No. 11 and the eluate was the material eluted in experiment No. 11. The antiserum was produced in rabbits by a series of intravenous injections of the toxin used in experiment No. 11.

In attempting to make the antisera, four rabbits were used, but in two of the rabbits, what was considered one-tenth of a rabbit LD_{50}, was injected and killed the rabbits. Therefore, a toxoid was used for the first injections and then toxin was used. One of the remaining rabbits was killed while bleeding it the second time to obtain serum. The antiserum of the other rabbit produced up to six lines of precipitation with type E toxin in the Ouchterlony test system.

Patterns obtained with closed strip paper electrophoresis with the eluate from experiment No. 11 and the type E toxin used in experiment No. 11 which had been dialyzed against barbital buffer, ionic strength 0.075 and pH 8.6, indicate the eluate moves toward the cathode slightly faster than one of the toxin fractions. The toxin formed four spots, two moving toward the cathode and two moving toward the anode. Other electrophoretic experiments gave the same number of fractions but the mobilities differed due to the changes in ionic strength of the buffer and the length of time the experiments were run.

V. DISCUSSION

This work has established that both type A and type E botulinus toxins can be adsorbed and a toxic fraction eluted from ion-exchange resins.

Elution from an ion-exchange resin has been proposed as a criteron of purity for proteins. The type E eluate fractions should be pure whether they are antigentically the same as the toxin molecule or have been changed by action of the ion-exchange resin.

Paper electrophoresis also indicates that the eluate is pure since only one component was found. The mobility of the eluate fraction is different from that of any component of the crude toxin. This shows that there has been some change in the toxin molecule that was eluted.

According to the results of the Ouchterlony test the eluate is not immunologically identical to any fraction of the crude type E toxin. This is possible, when the work with both type E and A toxins is examined critically.

The molecular weight of "pure" type A botulinus toxin is considered to be 900,000, therefore it is hard to believe that a molecule of that size could be readily absorbed from the intestinal tract. The difference between botulinus toxins and other toxins is that botulinus toxin is toxic by mouth and that toxicity is not destroyed by proteolytic enzymes of the intestinal tract. It is difficult to believe that these particular protein molecules

should be resistant to proteolytic enzymes. The assumption that toxin is not broken down is based on the fact that toxicity is not destroyed by pepsin or trypsin.

Therefore it may be possible for the eluate to contain a breakdown product of the type E toxin molecule and still possess toxicity.

The work at Fort Detrick by Duff et al. (34) shows that treatment of type E botulinus toxin with trypsin increases the titer of the toxin when it is inoculated intraperitoneally. The same toxin administered orally to mice shows no significant difference in toxicity. This certainly indicates that the proteolytic enzymes of the intestinal tract activate the type E toxin.

It would be very interesting to compare the molecular weight of purified type E toxin with the molecular weight of the purified toxin after treatment with pepsin, trypsin or papain. In fact, a similar study of type A and type B would be helpful in understanding this whole problem of toxicity of botulinus toxins.

Since one of the pressing problems concerning the study of botulinus toxins is their use in biological warfare, it would be highly significant to identify the smallest portion of the toxin molecule that still causes toxicity. It seems logical to assume that the large molecule of type A does not pass through the intestinal wall and that the size of the toxic fragment is quite small in comparison to the

original molecule. Of course the small fragments might combine after passage through the intestinal wall to again become toxic, for it seems that the toxin formed from bacteria must pass through the cell wall as small molecules and combine on the outside of the cell wall.

The alteration of the toxic portion of the toxin or a breakdown product of the toxin may be caused by secondary reactions taking place between the ion-exchanger and the toxin molecule. The acid or basic properties of the resin may cause some change in the toxin. This may be especially true with the strongly basic exchanger, Amberlite XE-98, since botulinus toxins are very labile in alkaline solutions. Also, the exposure to strong electrical fields of a strong-acid changer, Dowex 50, may be deleterious (47).

The different mobility of the eluate fraction as compared with the crude toxin indicates that there is a change in the net charge of the eluate molecule. It is usually considered that only the "surfaces" of the particles are involved in a change in mobility (48).

It should be remembered that a protein molecule does not have to be altered very much to change its antigenicity. Landsteiner has shown that just the addition of an acetyl, methyl or ethyl group to proteins shows a marked effect on the specificity of antigens.

Type E toxin must not be as labile as type A toxin to filtration, for filtration through a Selas 015 filter did

not significantly effect the toxicity.

This study has presented no evidence that type E botulinus toxin can be purified by ion-exchange resins. The writer is still of the opinion that with the number of new ion-exchangers that the chemists are making, one will eventually be found that can be used to purify type E botulinus toxin. The chemist and biologist are studying proteins more every day and therefore more ion-exchangers will be designed for work with proteins. In fact, some laboratories have started "tailor-making" polymers for specific uses (49).

Ion-exchangers should be of great interest to the microbiologist for they may eventually be used for purification of bacterial, rickettsial and virus toxins, and also many other metabolic products of microorganisms.

VI. SUMMARY

1. Crude type E botulinus toxin was adsorbed by a strong cationic exchanger, Dowex 50-X2 (H^+), and a toxic fraction was eluted from the exchanger with phosphate buffer, pH 7.0.

2. The eluate did not contain an antigenic component identical to any of those appearing in crude type E toxin.

3. The eluate moved as one component in electrophoretic experiments but did not have the same mobility as any of the components of crude type E toxin.

4. One of the components of the crude type E toxin reacted with normal serum to form a precipitate at 4 C but not at 37 C.

5. Filtration through a Selas 015 filter and storage in a freezer had no significant effect on the titer of crude type E toxin.

6. Crude type A botulinus toxin was adsorbed by a strong cationic exchanger, Dowex 50-X8 (H^+), and a toxic fraction was eluted from the exchanger with phosphate buffer at pH 7.0.

APPENDIX

Buffers Used in These Experiments

1. Phosphate buffer - pH 7.0

   39 ml of 0.4M monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$)

   61 ml of 0.4M dibasic sodium phosphate (Na_2HPO_4)

2. Phosphate buffer - pH 7.0

   39 ml of 0.2M monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$)

   61 ml of 0.2M dibasic sodium phosphate (Na_2HPO_4)

3. Acetate buffer - pH 4.1

   35 ml of 0.2M acetic acid

   10 ml of 0.2M sodium acetate

4. Acetate buffer - pH 4.2

   30 ml of 0.2M acetic acid

   10 ml of 0.2M sodium acetate

5. Acetate buffer - pH 4.6

   10 ml of 0.2M acetic acid

   10 ml of 0.2M sodium acetate

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