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Arthur W. Anderson

Polyacrylamide gel disc electrophoresis of concentrated culture media which had supported growth of Clostridium botulinum types A and B was shown to be effective in separating the toxin molecules from hemagglutinating molecules. Since the hemagglutinins of the two types are immunologically identical and are present in toxin and toxoid preparations, available antitoxins cross-react. The electrophoretic separation and elution of the toxins is observed as a possible means for future preparation of non-cross-reacting antitoxins.

The combination of a double-diffusion agar precipitin screening test applied to toxin-containing media and an immunoelectrophoretic toxin detection system demonstrates that the presence and

identity of a botulinal toxin in a substrate can be determined in vitro. Future application of the immunoelectrophoretic system described depends on availability of more specific non-cross-reacting antitoxins of high quality.

In Vitro Detection and Typing of Toxins Produced by *Clostridium botulinum* Types A and B by Immunoelectrophoretic Techniques

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[redacted]

Professor of Microbiology

in charge of major

[redacted]

Chairman of Department of Microbiology

[redacted]

Dean of Graduate School

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Typed by Illa W. Atwood for Glenn John Niedermeyer

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IN VITRO DETECTION AND TYPING OF TOXINS PRODUCED BY CLOSTRIDIUM BOTULINUM TYPES A AND B BY IMMUNOELECTROPHORETIC TECHNIQUES

INTRODUCTION

Detection and typing of the highly toxic proteins produced by the six known serotypes of Clostridium botulinum is presently accomplished by finding the antitoxin which neutralizes the toxin sample. Types A and B toxins and their antitoxins are quite specific as determined by mouse neutralization tests (17). A contribution to the development of a rapid and sensitive in vitro detection and typing system for the botulinal toxins is sought.

The ability to demonstrate the presence of the botulinal toxins by immunodiffusion reactions is well established (24). The sensitivity of this reaction with techniques used to date does not equal that of the animal test system (46). The problem of cross-reactions between the type A and B toxins and each other's type-specific antitoxin described by Lowenthal and Lamanna (32) also poses a problem of specificity in interpreting results obtained with the immunodiffusion reactions. At the same time, however, these authors established that the common hemagglutinin causing such cross-reactions and the non-cross-reacting toxin proteins were two distinct entities and that they could be separated from each other.

Rapid concentration and purification of proteins has been made

possible through disc ployacrylamide gel electrophoresis described by Ornstein (35) and Davis (15). The ability to apply such a system to protein toxin purification was demonstrated by Denny, Tan and Bohrer (16) with staphylococcal enterotoxin A.

An in vitro toxin detection system would be of great value in eliminating the necessity of toxin neutralization tests in animals. Also of considerable importance is the fact that toxins need not retain their biological activity to be detected by an in vitro immunological reaction.

The problem of concentrating crude toxin samples to increase sensitivity of detection reactions was approached by dialysis with hydrophilic polyethylene glycol.

An attempt to minimize the problems of specificity and sensitivity involved the separation of proteins in a sample by polyacrylamide gel disc electrophoresis to both further concentrate and isolate the toxins. The cross-reacting hemagglutinins associated with the type A and type B toxin preparations were separated from the non-cross-reacting toxins in this procedure.

Combining of electrophoretic separation and concentration of the toxins with a double-diffusion system was studied as an approach to an in vitro detection procedure.

LITERATURE REVIEW

Clostridium botulinum is a spore-forming, strictly anaerobic, rod-shaped bacterial species. Under as yet poorly understood conditions of growth it produces intracellularly a neurotoxin which is released into the growth medium after the major increase in the bacterial population has been achieved (26). Since the organisms are not capable of growing in the body of the warm-blooded animal, it is a matter of food poisoning by intoxication without infection.

The original case of food poisoning was described by van Ermengem (45) in 1896 and was caused by toxin produced in pickled ham. He isolated an anaerobic, spore-bearing, toxin-producing bacillus from the suspect ham which had caused several neuroparalytic illnesses and named the organism Bacillus botulinus. Twenty years later the Committee on Classification of Bacterial Types of the Society of American Bacteriologists recommended adoption of the generic term Clostridium for anaerobic bacilli showing a spindle-shaped enlargement at the site of spore formation (49).

Leuchs (31) in 1910 showed by means of equine antitoxins that two cultures arising from separate sources were morphologically and culturally almost identical but produced immunologically distinct toxins. At the present time there are six recognized types of C. botulinum, designated A through F, with each type producing an

immunologically distinct toxin. An excellent review of the history and development of botulism research appears in the U. S. Public Health Service publication, Botulism (43).

Numerous successful attempts at purification of the type-specific A and B toxins have been reported. Type A toxin was first concentrated by Snipe and Sommer in 1928 (40) by low pH isoelectric precipitation. Lamanna, Eklund and McElroy (27) in 1946 obtained the type A toxin in crystalline form by acid precipitation, shaking with chloroform and crystallization from ammonium sulfate solution. Abrams, Kegeles and Hottle (1), also in 1946, isolated the crystalline toxin without the chloroform treatment. Duff et al. (18) later reported on a simplified procedure for type A toxin isolation using acid precipitation, extraction in 0.075 molar calcium chloride, low pH ethanol precipitation and crystalization from ammonium sulfate solution. More recently the isolations of pure toxin have been combined with studies on the biochemical and biophysical properties of the molecules.

Clostridium botulinum type B toxin was first purified by Lamanna and Glassman (28) in 1947 by successive acid precipitations. Duff et al. (19) proposed a simplified procedure developed during studies on toxoid production.

The major problem at this time appears to be a disagreement on the molecular weight of the ultimate toxin molecules. Weights

varying between 900,000 (1, 30) and 13,000 (20) have been reported for type A toxins and weights between 60,000 (28) and 10,000 (22) for type B.

Considerable evidence exists to support the theory that the type A toxic protein of molecular weight 900,000 is an aggregate of several lower molecular weight toxic and non-toxic moieties.

Toxin dissolved in buffers at the pH range 6.5 to 8.0 and ionic strength 0.13 and above gradually dissociates into toxic units with molecular weights from 40,000 to 100,000 (38). Under conditions of mild alkalinity and high ionic strength, toxic fractions of the order of 150,000 can also be obtained (41).

Thermal inactivation curves indicate that the toxin splits into toxic subunits of two types, each having its own rate of inactivation (10).

When the toxin is subjected to guanidine acetate dialysis as a deaggregating procedure, a toxic subunit with a sedimentation coefficient of 0.93 and a diffusion coefficient of $6.128 \times 10^{-7} cm^2 sec^{-1}$ with a molecular weight 12,200 is obtained (20). Dolman's group feels that this molecule is the lowest common divisor of the toxin. Discrepancies in the amino acid composition found in Dolman's laboratory and that of Schantz's group at Fort Detrick lead the latter to believe the material with a molecular weight of 12,000 to 13,000 can not be the only protein subunit in the crystalline material. This

belief is supported by the evidence that the smallest repeating unit containing histidine has a molecular weight of 15,400, the smallest containing cystine, 27,400 and the smallest containing cysteine, 26,700 (41).

Research on the amino acid composition was originally performed on the type A toxin by Buehler et al. (9) who used microbiological assay procedures. Within the last three years the amino acid composition has been studied by several groups utilizing the modern chromatographic techniques (20, 41, 44). Although results are in general agreement, the information obtained by the various groups are not easily compared because each is working with a toxic unit of different molecular weight. There has been no unusual amino acid composition described which might help explain the extreme toxicity of the molecule. The amino acid composition of type B toxin has also been studied by one of these groups (22).

The origin of the toxins remains in dispute even in the light of two major attempts to settle the question. Bonventre and Kempe conducted a series of experiments on the physiology of toxin production by C. botulinum types A and B (3, 4, 5, 6). They indicated that during active growth the cells produce a "pro-toxin" that is non-toxic and that when autolysis occurs the proteolytic enzymes produced by the organisms attack the "pro-toxin" molecule and activate it. They further proposed that the reason type E toxin is activated

by trypsin is that the necessary proteolytic enzymes are not synthesized by type E cells as they are in the type A and B organisms.

The more recent observations by Gerwing (21) indicate that the toxin is synthesized in the latter stage of logarithmic growth and released into the culture medium upon autolysis. The data in this study agree with the results of previous work. This investigator, however, reports that a less toxic lower molecular weight intracellular toxin molecule varies in amino acid composition quite significantly from a more toxic higher molecular weight extracellular toxin in the culture lysate. The conclusion was reached that low molecular weight precursors aggregate to form the toxin molecules seen at autolysis. This is opposite of the mechanism proposed by Bonventre and Kempe.

In 1958 Boroff and Fitzgerald (8) proposed that loss of fluorescence could be used as a measure of loss in toxicity in the type A crystalline toxin. This was later shown to be invalid by Schantz, Stefanye and Spero (39) in 1960 when they demonstrated that either could be completely eliminated without affecting the other.

The antigen-antibody reaction of the toxins of C. botulinum has been discussed by several authors. The most enlightening studies are those of Lamanna and his associates (25, 29, 32, 33). In these publications the authors have demonstrated that the type A and type B toxins are both in close association with a common hemagglutinin

that causes the specific antitoxins of type A and B to cross-react. They further showed that the hemagglutinin and toxin molecules were two separate entities. The hemagglutinating activity of the toxin preparations has been studied by other investigators as well (23).

Immunological studies on vegetative cells of the botulinal organisms have been reported with the use of fluorescent antibodies. Batty and Walker found that fluorescent labeled antibodies could be used to differentiate cells of Clostridium septicum and Clostridium chauvei (2). They expanded their studies and reported that the same technique could be applied to distinguish between types A, B and F, types C and D, and type E cells of C. botulinum (48). They could not, however, distinguish between type A and type B cells using their system. Boothroyd (7) was able to produce specific fluorescent labeled antisera by agglutination-adsorption and reported in 1964 that the type A and B vegetative cells could be detected without cross-reactions.

Techniques of carrying out antigen-antibody reactions have been devised by Oudin (37), Ouchterlony (36) and Oakley (34). The double-diffusion agar precipitin technique developed by Ouchterlony was altered to a micro double-diffusion agar precipitin test by Wadsworth (47) in 1957. This was further simplified by Crowle in 1958 (14). The application of this system to the detection and study of staphylococcal enterotoxins was accomplished by Casman and his

associates (11, 12) and is now the accepted method for detection and typing of the enterotoxins in food samples. This replaces the antiquated kitten feeding test.

Double-diffusion agar precipitin techniques have recently been applied to the botulinal toxins (24, 42, 46) for toxin detection, but the cross-reactions occurring between type A and type B toxins still remain as a barrier to development of a practical system. At the present time the mouse protection tests are still regarded as the only acceptable means of toxin detection and typing.

MATERIALS AND METHODS

Microorganisms Used

Clostridium botulinum type A strain 33A and type B strain 115B were obtained from the Quartermaster Food and Development Command of the Armed Forces, Natick, Massachusetts. Clostridium botulinum type A strains 64-89, 73A and 78A and type B strains 32B, 113B and 213B were obtained from the National Canners Association, Washington, D. C. Clostridium botulinum type C strain 4218, type E strains VH, 4213, 4244, 4251 and 4299 and nontoxic type E strain 4267 were acquired from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland. Type F strain 202F C. botulinum came from the culture collection of the Department of Microbiology, Oregon State University, Corvallis, Oregon.

All strains were used in a modified Ouchterlony double-diffusion precipitin screening test for toxins. Strains of type A and type B were used in immunoelectrophoretic studies for detection and typing of toxins.

Culture Media

All C. botulinum stock cultures were stored at room temperature in the cooked meat medium proposed by Robertson consisting of

5.0 percent trypticase (Baltimore Biological Laboratories), 0.5 percent peptone (Difco), 1.0 percent glucose, distilled water to 1000 ml adjusted to pH 7.2 and three grams of raw ground beef per 10 ml liquid. The medium was prepared by adding the three grams of raw ground beef to 10 ml of the liquid in a 30 ml screw cap tube and was sterilized by autoclaving at 15 pounds steam pressure for 12 minutes.

Heavy surface inoculation of blood agar was used as a check for contamination. Blood agar was prepared by adding 10 percent sterile defibrinated sheep blood to blood agar base (Difco) which had been previously autoclaved for 15 minutes at 15 pounds steam pressure.

Yeast extract (YE) medium consisting of 2.0 percent yeast extract (Difco), 2.0 percent peptone (Difco), 1.0 percent glucose and distilled water to 1000 ml adjusted to pH 7.2 was used to grow all strains of C. botulinum for toxin production. Sterilization was accomplished by autoclaving for 15 minutes at 15 pounds steam pressure. The medium was utilized in the following volumes: 20 ml in a 30 ml screw cap tube, 500 ml in a 750 ml Erlenmeyer flask and 900 ml in a 1000 ml Erlenmeyer flask. All flasks were stoppered with cotton plugs.

Growth Conditions

Stock cultures were activated in 30 ml screw cap tubes containing 20 ml YE medium. Prior to inoculation the YE medium was heated to 100° C in a boiling water bath, held at that temperature for 10 minutes and shock cooled to 30° C in an ice-water bath to reduce the amount of oxygen dissolved in the medium. One ml of stock culture was then transferred into the bottom of the YE medium with a sterile, plugged, disposable Pasteur pipet. The inoculated tubes were incubated anaerobically at 30° C for 48 hours.

Toxin production was obtained by growing cells in 750 ml or 1000 ml Erlenmeyer flasks containing 500 and 900 ml YE medium respectively, or by growing cells in 30 ml screw cap tubes containing 20 ml YE medium. Following sterilization the medium was immediately shock cooled to 30° C in an ice-water bath and charged with a five percent inoculum from an 18 hour culture. Cultures were incubated anaerobically at 30° C for 72 hours.

Blood agar plates used for contamination checks were streaked with generous amounts of culture medium and incubated in the inverted position for 72 hours at 37° C.

Antitoxins

All C. botulinum type-specific antitoxins were obtained from the United States Public Health Service, Communicable Disease

Center, Atlanta, Georgia. The antitoxins were prepared from equine serum and received in the lyophilized state. Types A, B, C and E antitoxins were reconstituted with sterile distilled water to a concentration of 10 International Units (IU) per ml serum. Type F antitoxin was reconstituted as above to contain eight IU per ml serum. These antisera were stored at 5° C and used in mouse toxicity tests for checking identity of the cultures. Types A, B, E and F antitoxin were used for in vitro immunological reactions with the botulinum toxins.

Identification

Purity of cultures was checked by microscopic examination of Gram stains and by aerobic incubation of blood agar with heavy surface inoculum from culture medium.

The identities of the C. botulinum types were checked in mice by toxin neutralization tests. For the neutralization test, 0.25 ml of specific antitoxin was mixed in a disposable 2.5 ml syringe with 0.25 ml of toxin-containing medium diluted 1:10 with sterile 0.85 percent saline. The syringe was left at room temperature for one hour prior to injection. Three 20 gram, male white mice were injected intraperitoneally with 0.5 ml of toxin-antitoxin mixture and three mice were injected via the same route with 0.25 ml of the 1:10 dilution of the toxin-containing medium. The mice were

observed for 48 hours. Those mice demonstrating labored breathing followed by death were assumed to have died from the botulism toxin. The type of specific antitoxin affording protection from terminal symptoms indicated the type of C. botulinum culture in question.

Toxin Screening Test

Initial testing for botulinum toxin in culture medium was accomplished by double-diffusion agar precipitin technique. The screening tests were performed on clean 76 mm X 25.4 mm standard glass microscope slides coated with 0.2 percent Ionagar No. 2 (Oxoid) in distilled water as described by Crowle (14). Ionagar No. 2 at a concentration of 1.0 percent was dissolved in 0.05 molar phosphate buffer at pH 7.1 containing merthiolate (1:10,000). Four ml of 1.0 percent agar at 45° C was applied to each coated slide and allowed to cool.

The template used for this screening test is shown in Figure 1. An antitoxin trough 63 mm X 1 mm was cut into the agar five mm from the edge of the slide. Five wells, each five mm in diameter, were placed in a line parallel to the trough at a distance of four mm from the trough with an eight mm space between each well.

Each trough received 0.2 ml (2 IU) of the C. botulinum type B antitoxin. One-tenth ml of known type B toxin-containing medium

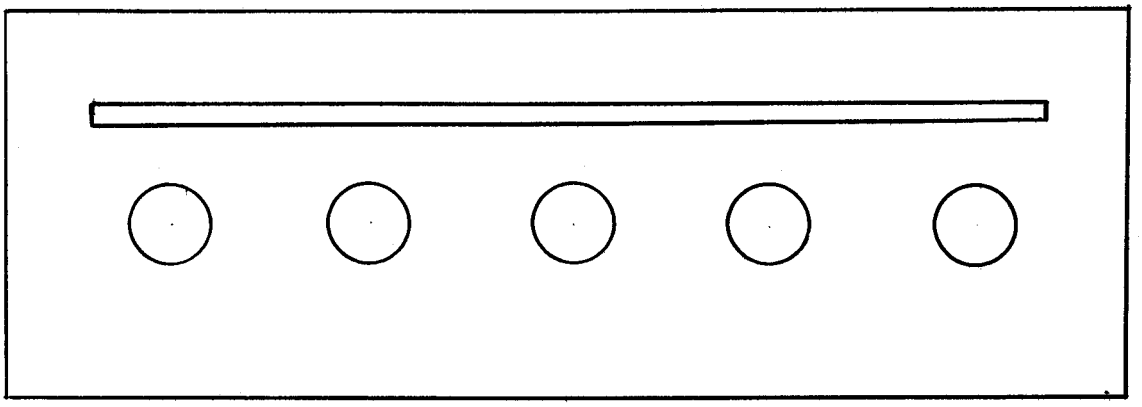

Figure 1. Template for toxin screening test (2X).

was placed in one of the wells as a positive control and 0.1 ml of sterile uninoculated medium was placed in a second well as a negative control. The three remaining wells each received 0.1 ml of growth medium to be tested. The slides were then placed in a moist chamber at 25° C for 24 hours. At the end of 24 hours the slides were removed from the moist chamber and presence or absence of precipitin lines was noted.

For permanent recording of slide reactions, the agar slides were washed with 0.85 percent saline for 24 hours to remove non-precipitated protein. Following this wash the agar slides were placed in a solution of 2.5 percent trichloroacetic acid (TCA) for five minutes. They were then transferred to a solution of 0.5 percent light green SF in 5.0 percent TCA for one hour and stored in 5.0 percent TCA until the immunoprecipitins could be recorded on Kotabromide F-5 Photographic Paper using a Kodak Model-2 Precision Enlarger.

Concentration

Following anaerobic incubation the cells were cleared from the culture medium by centrifugation at 10,000 X g for 20 minutes at 4° C. Filtration through an HA Millipore filter removed any residual cells. The filtrates were concentrated by dialysis in cellulose tubing against 50 percent (weight/weight) Polyethylene Glycol, 4000

in water at 5° C. After eight hours the concentrated toxin-containing medium was transferred to sterile 30 ml screw cap tubes and stored at 5° C. The concentrates were used in the immunoelectrophoretic toxin detection system.

Electrophoresis Solutions

The ingredients for preparation of stock solutions used in the polyacrylamide electrophoresis are as follows:

Acrylamide Monomer (Eastman Organic Chemicals)

N,N'-Methylenebisacrylamide, Bis (Eastman Organic Chemicals)

2-Amino-2-Hydroxymethyl-1,3-Propandiol, Tris (Eastman Organic Chemicals)

N,N,N',N'-Tetramethylethylenediamine, Temed (Eastman Organic Chemicals)

Ammonium Persulfate (Baker and Adamson)

Glycine, Ammonia free (Sigma)

1 N Hydrochloric acid

Kodak Photo-Flo 200 Solution

Bromphenol Blue (Matheson, Coleman and Bell)

The stock solutions prepared from the above ingredients are as follows:

(A) 1 N HCl 48 ml
Tris 36.3 g
Temed 0.23 ml
H_2O to make 100 ml
(pH 8.8 - 9.0)

(H) Buffer 10X (as used)

Tris 3.0 g
Glycine 14.4 g
H_2O to make 1000 ml

(C) Acrylamide 28.0 g
Bis 0.735 g
H_2O to make 100 ml

(G) Catalyst

$(NH_4)_2S_2O_8$ 0.14 g
H_2O to make 100 ml

(J) Tracking Dye

0.005% Bromphenol Blue in H_2O

Solution 1

1 Part (A)
2 Parts (C)
1 Part H_2O
(pH 8.8 - 9.0)

The working solutions for polyacrylamide electrophoresis are as follows:

Separating Gel Solution

1 Part Solution 1
1 Part Solution G

Stacking Gel Solution

1 Part Upper Gel (Canalco)
1 Part H_2O or Sample

Electrophoresis

The disc gel electrophoresis method described by Ornstein (35) and Davis (15) was used in this study. Five mm glass tubing was sectioned into gel supports 63 mm in length. The 63 mm X 5 mm

tubes were rinsed in Kodak Photo-Flo 200, diluted 1:200 in distilled water, and allowed to dry in an upright position. The tubes were then inserted into fixed, inverted rubber caps which closed-off the bottom ends and held the tubes in a vertical position. Fresh Catalyst Solution G was prepared prior to each experiment. Separating Gel Solution 1 and the Catalyst Solution G were raised to room temperature. The Separating Gel liquid was prepared by mixing equal volumes of the two solutions.

Liquid Separating Gel Solution was added to the glass tubes to within 12 mm of the upper end with a glass syringe. The gel solution was overlaid with distilled water and the gel allowed to polymerize at room temperature for 40 minutes. Following polymerization the liquid layer consisting of the distilled water and oxygen-inhibited monomer solution was removed with a disposable Pasteur pipet.

Upper Gel Solution (Canalco) was used to rinse the area above the polymerized Separating Gel. Stacking Gel Solution was prepared by mixing equal volumes of Upper Gel and distilled water. A six mm column of Stacking Gel Solution was then applied on top of the Separating Gel and overlaid with distilled water. The gel was photopolymerized in the presence of a 15-watt fluorescent lamp placed approximately 20 mm above the tubes. Polymerization time was 15 minutes. Upon photopolymerization of the six mm column of stacking gel, the distilled water layer was removed with a disposable

Pasteur pipet.

Equal volumes of toxin-containing medium concentrate and Upper Gel Solution were mixed at room temperature. The surface of the gel column was rinsed with Upper Gel Solution and 100 µl of the above sample gel solution was added to form a sample column six mm in depth. This sample gel was photopolymerized by the same procedure as was the stacking gel. When a sample gel failed to polymerize the toxin-containing medium concentrate was diluted 1:10 with distilled water prior to mixing with an equal volume of Upper Gel Solution. The tubes were removed from their preparation supports and placed in the electrophoretic cell with the sample gel at the top.

Tris-glycine buffer solution was diluted 1:10 with distilled water and both upper and lower reservoirs were filled with the diluted buffer to within 10 mm of the reservoir tops. One-tenth ml of 0.005 percent bromphenol blue tracking dye solution was placed in the upper reservoir for every 100 ml buffer. Electrophoresis was performed in the upright position with the current adjusted to 2.5 milliamperes per tube. Electrophoresis was terminated when the dye front had migrated to within five mm from the bottoms of the separating gels (between 45 and 60 minutes). The protein-laden polyacrylamide gels were removed from the tubes under water by rimming with a needle. The gels were immediately placed in a

cutting block and sliced longitudinally into two equal sections with a razor edge. The gel halves were either stained for total protein, frozen immediately and stored at -20° C or placed in a trough parallel to a reservoir containing type B antitoxin in an agar gel slide.

Total Protein Staining

The procedure used to stain protein zones in the polyacrylamide gels was that described by Chrambach, et al. (13).

Following electrophoresis the protein-laden gel halves were suspended in 18 mm X 150 mm glass tubes containing 25 ml each of 12.5 percent TCA. After 30 minutes they were immersed in staining solution freshly prepared by a 1:20 dilution in 12.5 percent TCA of a 1.0 percent aqueous stock solution of Coomassie blue (Colab Laboratories Incorporated). The gels remained in the staining solution for one hour and were then transferred to 10 percent TCA. At this time the protein bands were visible and continued to gain in intensity over a 48 hour period.

Localization of Immunologically Reactive Zones

The zones in the polyacrylamide gels which contained proteins capable of reacting immunologically with specific antitoxin were located through the use of an acrylamide gel-agar double-diffusion slide precipitin test.

Standard 76 mm X 25.4 mm microscope slides were cleaned and coated with a thin layer of 0.2 percent ionagar as previously described. Four ml of 1.0 percent ionagar in phosphate buffer, prepared as described previously, was applied to each slide and allowed to cool. An antitoxin trough 60 mm X 1 mm was cut into the agar five mm from the edge of the slide. Another trough measuring 50 mm X 3 mm was cut into the agar parallel to the antitoxin trough at a distance of four mm from that first trough. The agar was removed using a narrow spatula and the protein-laden polyacrylamide gel was laid into the resulting trough. Caution was exercised to assure that intimate contact was obtained between the flat edge of the polyacrylamide gel half and the agar separating the gel from the antitoxin. Two-tenths ml of type B specific antitoxin (2 IU) was placed in the antitoxin trough and the slides placed in a moist chamber at 25° C for a period of 18 hours to 24 hours. Precipitin lines formed in the agar were compared with the protein bands in the gel by removing the polyacrylamide gel half from the trough and replacing it with its corresponding gel half which had been stained for total protein.

Results were photographed with a Poloroid MP3 technical camera using transmitted and reflected light.

Toxicity of Immunologically Reactive Zones

The toxicity of protein zones in the polyacrylamide gels that were shown to produce precipitin lines in the double-diffusion slide precipitin test with type B specific antitoxin were analyzed qualitatively in mice.

Frozen gel halves were compared with precipitin lines formed by proteins from their corresponding gel halves. One mm sections of the frozen polyacrylamide gels directly opposite the centers of arcs of the precipitin lines were cut out with a razor and placed in conical centrifuge tubes. Each gel was macerated in the presence of 1.8 ml of 0.05 molar phosphate-buffered 0.85 percent saline at pH 6.8. Maceration was accomplished with a two mm solid glass rod and the gel pieces adhering to the rod were rinsed into the tube with an additional 0.2 ml of the above buffer. Elution of the protein from the broken polyacrylamide gels was completed by storage at 5° C for a period of 12 hours.

Following the elution of the acrylamide gels an additional three ml of buffered saline was introduced into the tubes and the contents stirred with a glass rod. The liquid was then separated from the acrylamide pieces by filtration through Whatman GF/A Glass Paper (W. and R. Balston Limited). Each of three 20 gram male white mice received an intraperitoneal injection of 0.5 ml filtrate. Three

additional mice received intraperitoneal injections of 0.5 ml of a 1:1000 dilution of the filtrate diluted with the same solution used for elution of the gels. Two mice were injected intraperitoneally with 0.25 ml filtrate mixed with 0.25 ml specific antitoxin after the combination had been allowed to incubate at room temperature for one hour. Two mice were injected intraperitoneally with 0.5 ml filtrate from maceration of a polyacrylamide gel to which no sample had been applied. The mice were observed for a period of 72 hours for terminal symptoms of botulism.

Hemagglutinating Activity of Immunologically Reactive Zones

A qualitative test for hemagglutinating activity was conducted on those protein bands in the polyacrylamide gels which demonstrated immunological activity toward the type B specific botulinal antitoxin in vitro.

Sterile, defibrinated sheep blood was centrifuged to remove the cells at 2,000 X g at 5° C and the erythrocytes were washed six times with 0.85 percent saline at pH 7.0. The washed erythrocytes were again packed by centrifugation and the packed cells were considered a 100 percent suspension. A 2.0 percent suspension of sheep erythrocytes was then prepared using 0.85 percent saline as the suspending medium.

Immunologically reactive protein zones in polyacrylamide gels

were located and eluted by the same procedure used to determine the toxicity of these proteins with the exception that the pH of the eluent in this case was 7.0 rather than 7.5.

The hemagglutination tests were performed in 12 mm X 75 mm glass tubes. Two ml of the 2.0 percent sheep erythrocyte suspension was placed in each tube. An equal volume of protein-containing eluent was added to the erythrocyte suspension with duplicates for each sample. Two control tubes were prepared with two ml eluent from macerated acrylamide gels, to which no sample was applied, and two ml 2.0 percent erythrocyte suspension. The mixtures were gently triturated with a five ml pipet and allowed to react at 5^{o} C for two hours. Hemagglutination was observed under ten power magnification with a stereomicroscope.

RESULTS

The results obtained with all type A strains of C. botulinum tested were identical as were the results of all type B organisms. The media in which growth of toxic C. botulinum strains occurred were all shown to contain biologically active toxin.

The quality of type A specific antitoxin precluded its use in precipitation reactions. Numerous attempts to obtain precipitin lines between type A toxin-containing media and type A antitoxin yielded negative results.

Toxin Screening Test

Unconcentrated toxin-containing media which had supported growth of C. botulinum type A strains exhibited one precipitin line when reacted with type B specific antitoxin in the screening test. Strains of C. botulinum type B all formed two precipitin lines in the same system. Typical reactions of type B strains are shown in Figure 2. Single and double precipitin lines associated with type A and type B strains respectively are shown in Figure 3. Sterile medium produced no precipitin lines in the screening test with the type B antitoxin. Precipitin lines were likewise not produced by toxin-containing media which had supported growth of C. botulinum

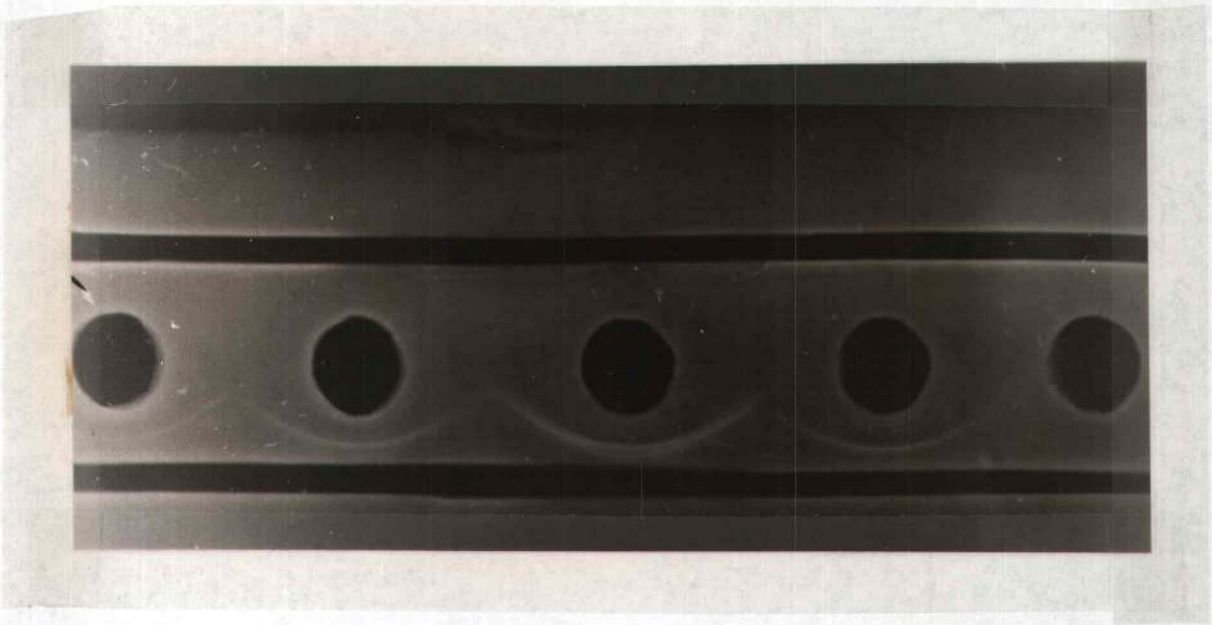

Figure 2. Double-diffusion agar precipitin reactions between type A antitoxin (top trough), type B antitoxin (bottom trough) and various strains of type B C. botulinum lysate. Left to right, 32B, 113B, 115B, 213B and sterile, uninoculated YE medium.

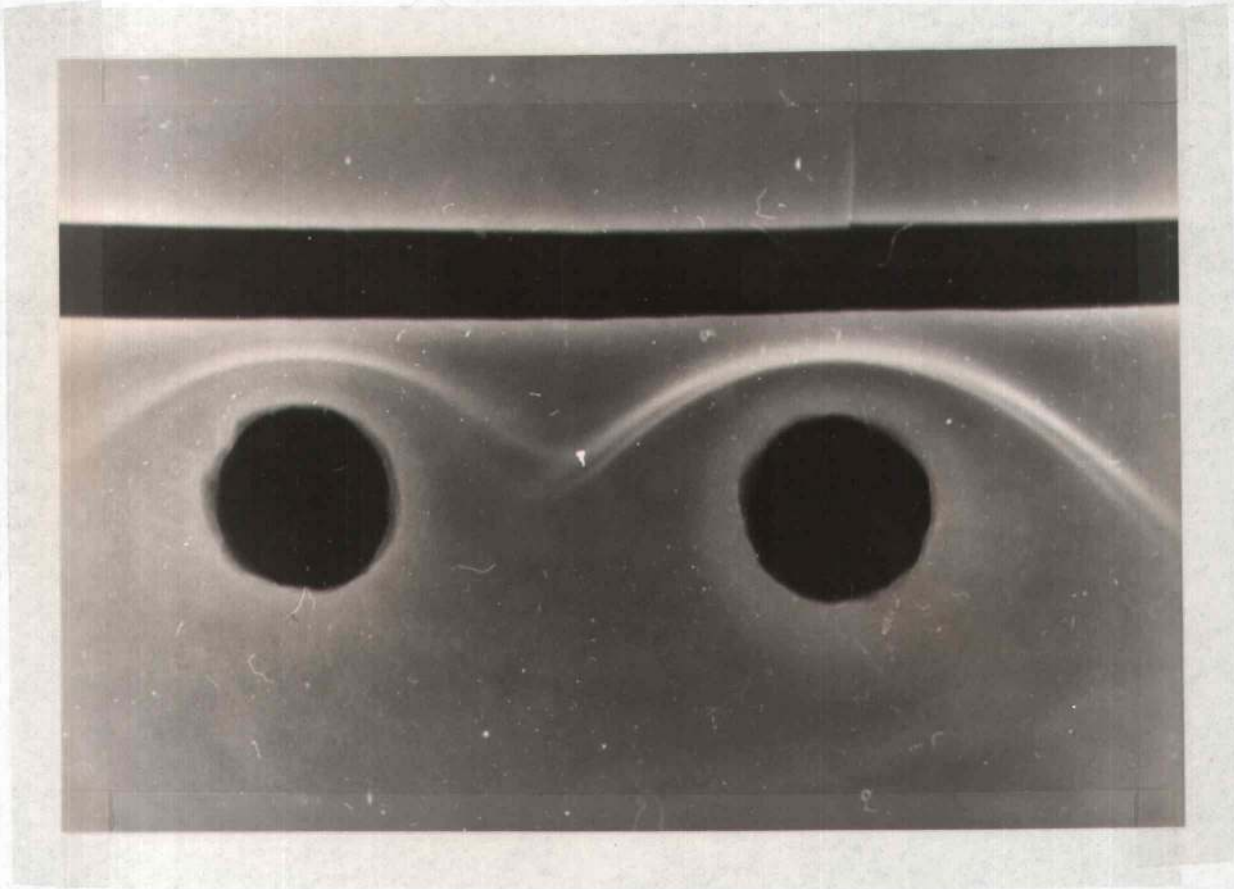

Figure 3. Double-diffusion agar precipitin reaction between type B antitoxin (trough) and type A and B C. botulinum lysates. Left to right, 33A and 115B.

types C, E or F.

Precipitin lines were formed consistently by media which had supported growth of type A and type B organisms as early as eight hours after inoculation of the culture media. Although toxin was detected by this system four hours after inoculation of type B strains 115B and 213B, these results were not consistently reproducible. Toxin-containing media of all strains of type A and type B stored up to 120 days at 5° C produced results identical to freshly obtained toxin-containing media.

Both concentrated and unconcentrated media which had supported growth of type C organisms failed to produce precipitin lines when tested with type C antitoxin although presence of toxin was established by intraperitoneal injection of mice.

Concentrated type E toxin-containing medium consistently gave rise to one precipitin line with type E specific antitoxin and did not cross react with type F specific antitoxin, Figure 4. Unconcentrated type E toxin-containing media failed to react with the specific antitoxin under the same conditions. Nontoxic type E strain 4267 medium failed to give a precipitin line with type E antitoxin prepared from a toxic type E strain, Figure 5.

The type F strain of C. botulinum yielded one precipitin line from both concentrated and unconcentrated toxin-containing media with type F antitoxin and did not cross-react with type E antitoxin,

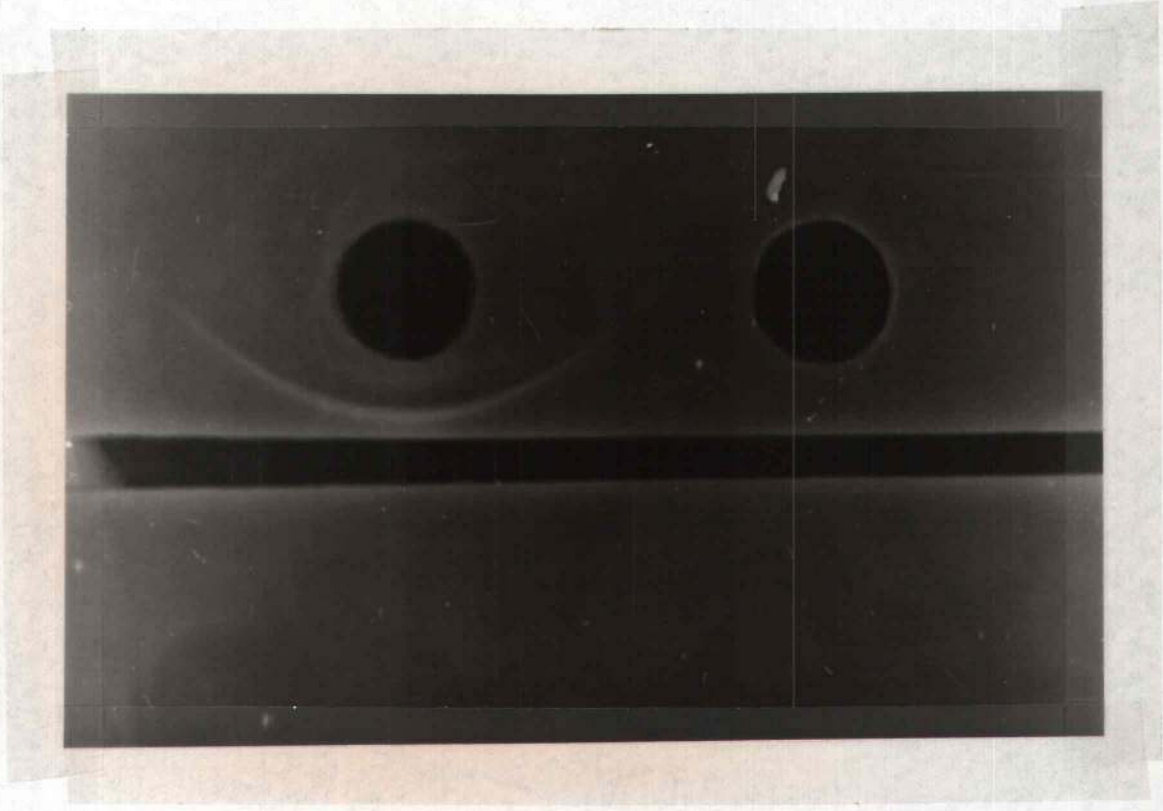

Figure 4. Double-diffusion agar precipitin reactions between C. botulinum type E toxin-containing medium (trough), type E antitoxin (left) and type F antitoxin (right).

Figure 5. Double-diffusion agar precipitin reactions between C. botulinum type E antitoxin (trough), type E toxic strain VH lysate (left) and type E non-toxic strain 4267 lysate (right).

Figure 6. Reactions of C. botulinum type C, E and F strains were not pursued beyond this point in this particular study.

Unconcentrated toxin-containing medium of type A strain 33A incubated for 72 hours gave a single precipitin line after being serially diluted with 0.85 percent saline to a dilution of 1:64 when reacted with type B antitoxin. These results were reproducible in five subsequent tests.

Unconcentrated toxin-containing medium of type B strain 115B incubated 72 hours gave two precipitin lines when diluted as above to a dilution of 1:512 and tested with type B antitoxin. These results were likewise reproducible.

A series of 25 coded unknown cultures in 20 ml YE medium was screened 18 hours after inoculation. Fourteen of the 25 cultures gave positive reactions in the screening test with type B antitoxin after 12 hours. Comparison of code numbers with the preparation chart showed that all 14 strains which produced one or two precipitin lines were type A or type B cultures. The remaining 11 strains were type E and F cultures of C. botulinum. Since a a minimum of 24 hours was occasionally necessary for appearance of the second precipitin line in the case of the type B cultures, no attempt was made to separate type A or type B cultures at this point on the bases of the number of precipitin lines present.

The occasional appearance of a precipitin line closer to the

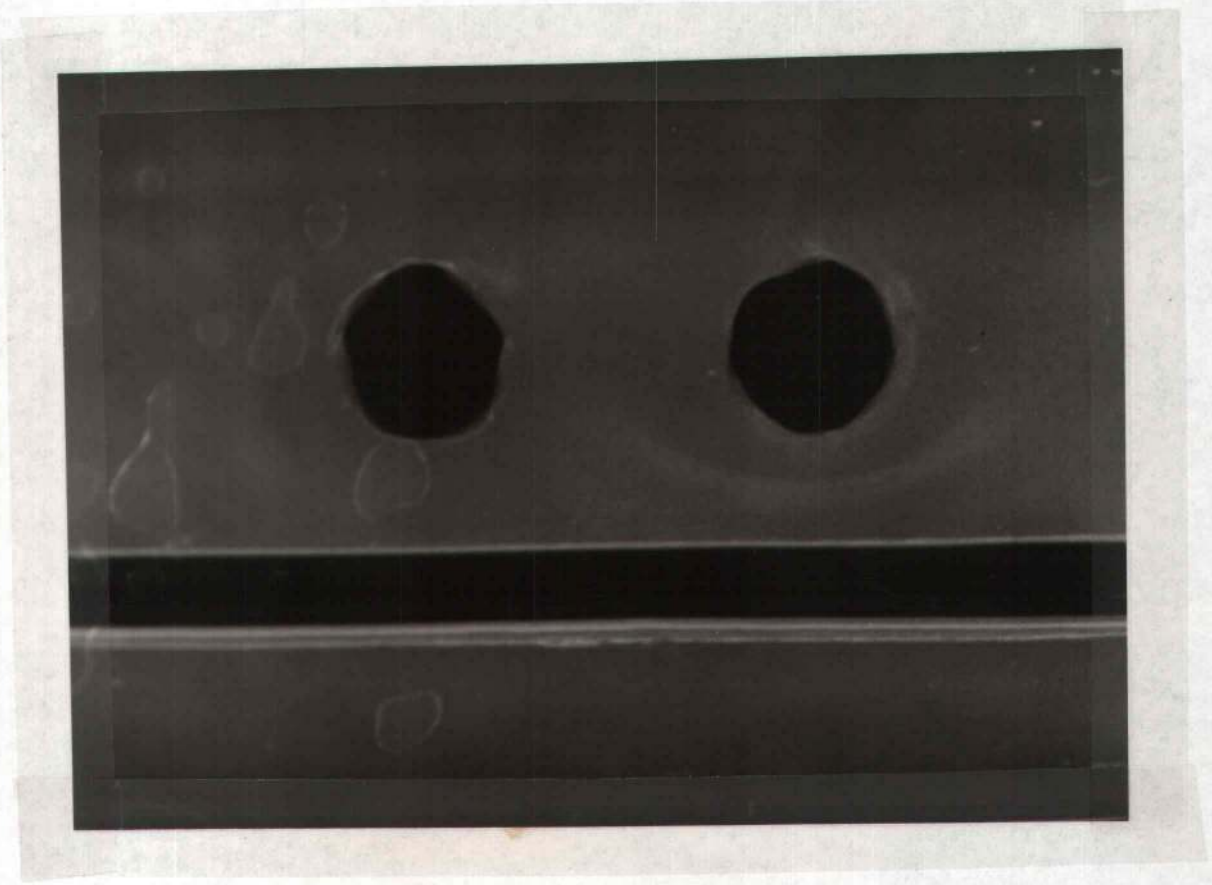

Figure 6. Double-diffusion agar precipitin reaction between C. botulinum type F strain 202F lysate (trough), type E antitoxin (left) and type F antitoxin (right).

toxin origin than those discussed was noted with unconcentrated type A and type B samples. This weak precipitin line was not observed in samples which had been concentrated by dialysis. Furthermore, this precipitin line frequently disappeared within 24 hours after the start of the immunodiffusion reaction and was not present after 48 hours in any of the experiments.

All cultures were incubated an additional 12 hours during which time the double-diffusion precipitin reactions were occurring. Those cultures yielding positive results were cleared of cells, after a total of 30 hours incubation, and the media were concentrated. Concentration of 20 ml of medium resulted in 0.4 to 0.8 ml of sample. The concentrated media were then subjected to the immunoelectrophoretic system for typing.

Immunoelectrophoresis of Toxin-Containing Media

Electrophoresis in polyacrylamide gels of the concentrated toxin-containing media of type A strain 33A and type B strain 115B resulted in separation into from 17 to 20 protein zones. Gels stained for total protein are shown in Figure 7. One common band (HA) was later shown to be a protein capable of *in vitro* immunological reaction with type B antitoxin and to possess hemagglutinating activity while totally lacking in toxicity.

Electrophoretically separated proteins of type A media in

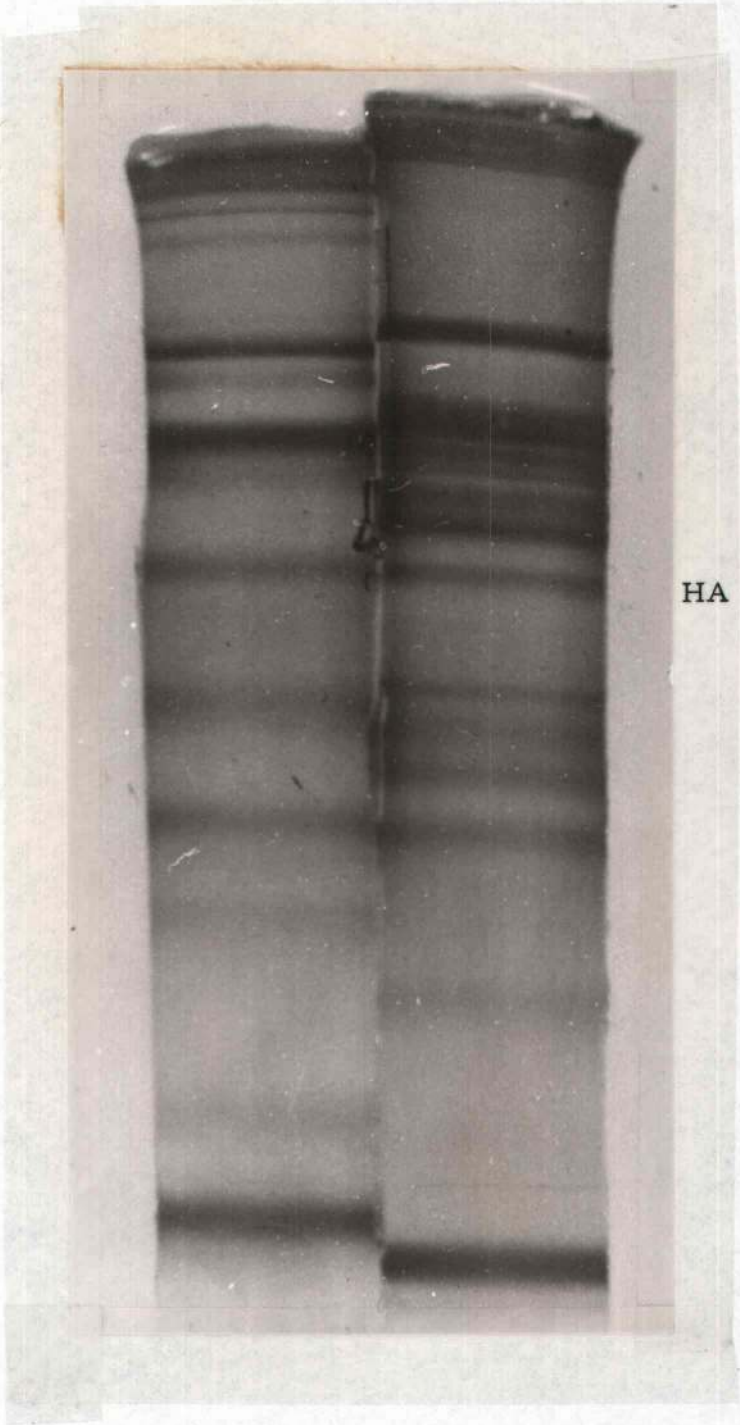

Figure 7. Total protein stained gels showing patterns of strains 33A (left) and 115B (right) of C. botulinum lysate. Common hemagglutinin protein zone is indicated (HA).

acrylamide gels reacted with type B antitoxin across a four mm agar strip resulted in formation of one precipitin line, Figure 8. Gels containing type B separated proteins yielded two precipitin lines, Figure 9. The lower precipitin line of the type B reaction corresponded to the single precipitin line formed in the type A reaction. These results were completely reproducible on more than 20 separate occasions. Toxin-containing media stored for as long as 120 days at 5° C and then concentrated gave reactions identical to those of freshly prepared samples. Precipitin lines in all cases were visible within eight hours. Each experiment consisted of either eight or sixteen samples.

The 14 coded unknown cultures which gave positive reactions in the toxin screening test were subjected to the immunoelectrophoretic procedure. Eight gave one precipitin line identical to the reaction seen in Figure 8. Six produced two precipitin lines identical to the reaction seen in Figure 9. Comparison of code numbers with the preparation chart showed that all type A and type B cultures were correctly identified.

Time necessary for typing of the toxin present in a liquid medium which has supported growth of type A or type B cells of C. botulinum was approximately 22 hours (12 hours for the screening test and 10 hours for electrophoresis and immunodiffusion).

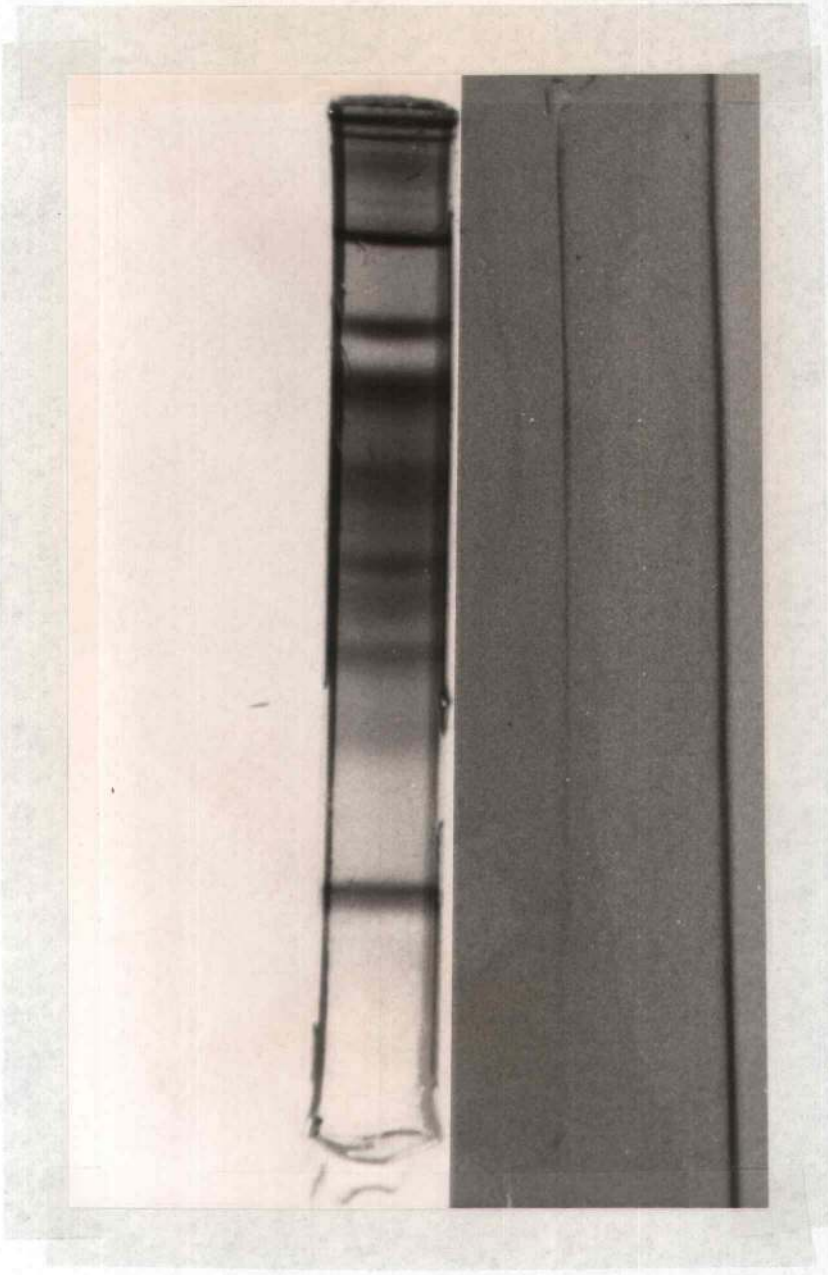

Figure 8. Single precipitin line resulting from double-diffusion reaction of type A C. botulinum lysate in acrylamide gel and type B antitoxin. Stained gel half corresponds to gel half used in reaction.

Figure 9. Precipitin lines resulting from double-diffusion reaction of type B C. botulinum lysate in acrylamide gel and type B antitoxin. Stained gel half corresponds to gel half used in reaction.

Toxicity of Proteins

The single protein zone which formed a precipitin line with type B antitoxin when type A toxin-containing medium had been electrophoresed was located, eluted and tested for toxicity in mice. None of the unprotected six mice receiving intraperitoneal injections developed symptoms of botulism. The two protected mice and two control mice remained healthy and active. The same results were obtained upon testing of the lower immunologically reactive zone in the type B sample electrophoresed on acrylamide gel. Both of these proteins occupied a similar position in their respective gels.

The type B protein producing the upper precipitin line with type B antitoxin was tested for toxicity. All six unprotected mice developed symptoms of botulism and died within 36 hours. The two mice protected with type B antitoxin and the two control mice remained healthy and active.

Hemagglutinating Activity of Proteins

The protein(s) in type A and B toxin-containing media capable of immunological reaction with type B antitoxin were eluted and tested for hemagglutinating activity. Both the lower immunologically reactive protein band in the type B acrylamide gels and the single immunologically reactive band in the type A acrylamide gels

possessed hemagglutinating activity as demonstrated with sheep erythrocytes at 5°C. The upper protein band in the type B acrylamide gel which was precipitated by the type B antitoxin and was shown to be toxic failed to agglutinate sheep red blood cells under the same conditions. The hemagglutinating protein zones in type A and type B acrylamide gels were eluted and reacted against type B antitoxin in a modified Ouchterlony double-diffusion system. The result was a line of identity produced by the two eluents, Figure 10.

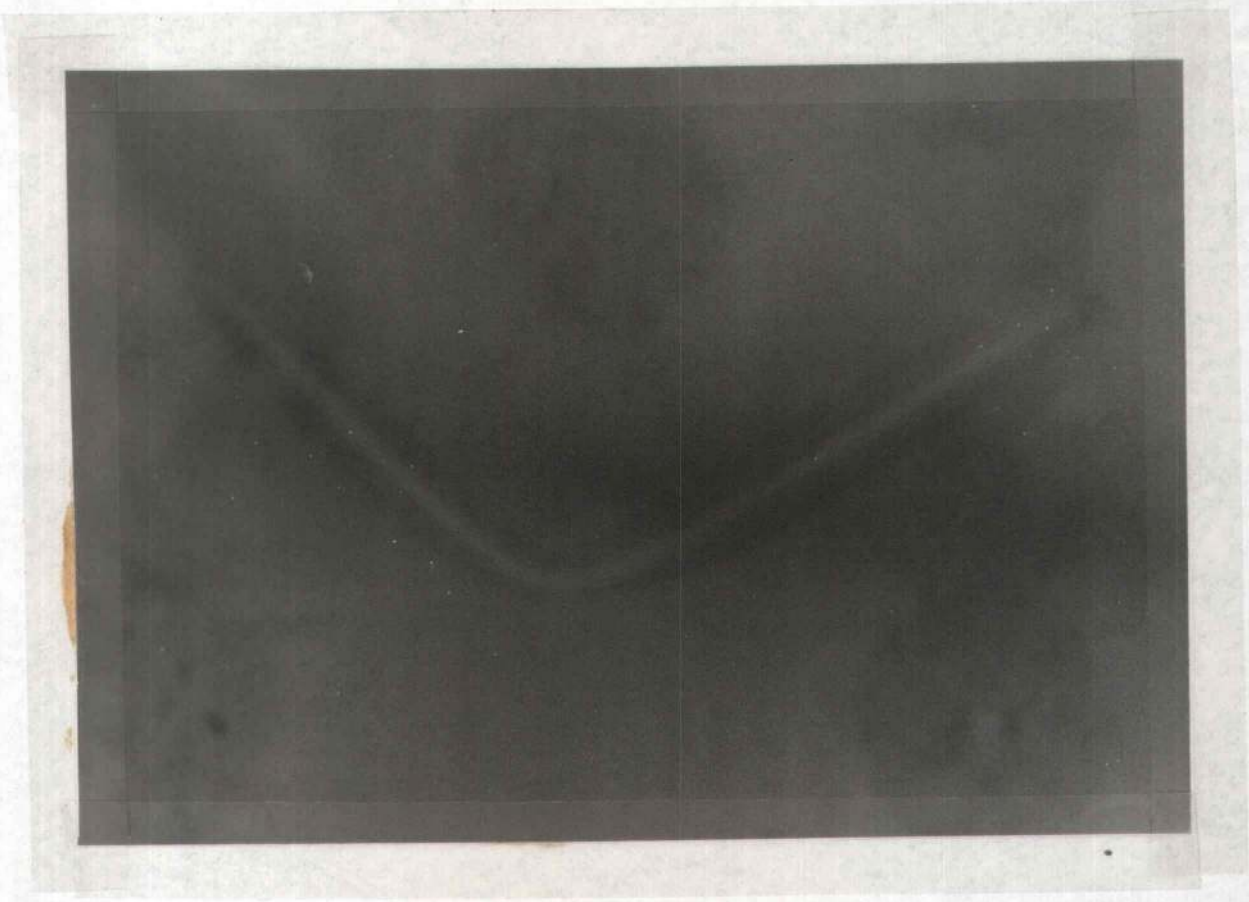

Figure 10. Reaction of hemagglutinating protein eluted from C. botulinum type A acrylamide gel (left), hemagglutinating protein eluted from type B acrylamide gel (right) and type B antitoxin (top). Line of identity is observed.

DISCUSSION

The results obtained with the double-diffusion agar precipitin screening test are in agreement with the results of other investigators (24, 46) which were published after this portion of my own research had been completed. One of these authors (24) also found the Communicable Disease Center type A antitoxin to be incapable of precipitating type A toxin. These results are also in agreement with the findings of Lamanna and his associates concerning the cross reactions of the antitoxins in the in vitro system.

Since the quality of the type A antitoxin available precluded its use in this system, the type B antitoxin cross-reaction with type A toxin was an important asset in the indirect detection of that protein.

The sensitivity of this system is apparently quite high since only eight hours of incubation was sufficient to detect toxin in the unconcentrated culture media. At eight hours very little toxin is actually present in the culture medium as shown by Bonventre and Kempe (5). No crystalline toxin was available to me during this investigation; therefore, a true quantitative estimate of sensitivity was not possible.

The results of this phase of research do show that a simple in vitro test for presence of botulinal toxins would require a more

specific antitoxin prepared from a toxoid free of the cross-reacting hemagglutinin.

The results of the immunoelectrophoretic technique are, I believe, more significant. Lamanna, in a review of the state of botulism research, stated that a convenient method of separating the hemagglutinin from the toxins would be of great value (26). The results of my investigation suggest that through polyacrylamide gel electrophoresis the two molecules can indeed be not only separated but simultaneously concentrated in a manner which lends itself to easy recovery of the two proteins. The use of such a system in preparation of toxoids could possibly lead to the availability of non-cross-reacting antitoxins on a large scale.

I do not envision this system as a toxin typing technique in itself, but the fact that type A and type B toxins can be differentiated by this system indicates that it is a step in the right direction. When non-cross-reacting antitoxins of sufficient quality do become available, the application of the immunoelectrophoresis technique described here which concentrates, separates and partially or totally purifies the toxins and presents them in an easily detectable form could prove to be the most acceptable test system. This system which will require a more specific antitoxin of higher quality may prove to be the means of obtaining that higher quality of antitoxin which the system itself requires.

Another possibility is the detection of the toxin zones in polyacrylamide gels with fluorescent antibodies. This would further increase the sensitivity and rapidity of the detection scheme.

Someday, perhaps soon, a rapid, sensitive and specific *in vitro* detection system for botulinal toxins will replace the use of animals just as an *in vitro* system has replaced the use of kittens in testing for staphylococcal enterotoxins.

SUMMARY

Culture media which had supported growth of Clostridium botulinum cells were subjected to an in vitro immunological detection system. Double-diffusion agar precipitin testing of the samples with specific antitoxins indicated that the presence of these toxins could be detected in vitro. Cross-reactions occurring as a result of a common hemagglutinin shared by type A and type B culture lysates resulted in multiple precipitin lines in a type B homologous system and a single precipitin line in a type A heterologous system.

Concentrated toxin-containing media were subjected to polyacrylamide gel disc electrophoresis. It was shown that the toxin and hemagglutinin could be separated, and the toxin could be detected by a polyacrylamide gel-agar double-diffusion precipitin technique. A test of the ability of this system to differentiate between type A and type B toxin-containing media demonstrated that the specific type of toxin present could be accurately determined.

The results suggest that polyacrylamide gel electrophoretic separation of the toxins could be used as a tool in preparing non-cross-reacting antitoxins. With the availability of more specific antitoxins, this system, or variations of the system, could prove to be the most acceptable toxin detecting and typing procedure.

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