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Detection of Botulinal Toxins by Immunodiffusion[1]

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A procedure employing concentration with Sephadex and analysis by gel diffusion (Ouchterlony) was used to detect the toxins of *Clostridium botulinum* in foods. Botulinal toxins with toxic levels of 370 to 557 mouse LD_{50} per milliliter were detected in the food samples. Test results were verified by use of the mouse protection test. Approximately 24 hr were required to complete the entire procedure.

Botulism is a very rare, but highly fatal, disease for which no adequate clinical diagnostic test is available. At present, the most effective method of corroborating the diagnosis of botulism is to demonstrate that the filtrate of a suspected food sample contains botulinal toxin and will kill mice when inoculated into them. Mice and other animals have limitations which prevent them from being perfect tools for botulism studies.

Botulinal toxins are simple proteins and can be demonstrated in vitro by immunological procedures. A hemagglutination procedure sensitive enough to detect one LD_{50} mouse unit of botulinal toxin was recently reported by Johnson et al. (7). Immunodiffusion methods have been shown by Casman and Bennett (4) and by Read et al. (8) to detect small quantities of proteinaceous toxins such as staphylococcal enterotoxins in foods. The work reported here attempts to adapt the gel-diffusion technique to the detection of botulinal toxins in foods.

MATERIALS AND METHODS

Sources of toxins. Purified, crystalline, type A botulinal toxin was obtained from E. J. Schantz, U.S. Army Biological Laboratory, Fort Detrick, Md.

Clostridium botulinum type A, #26, *C. botulinum* type B, #20, and *C. botulinum* type E, #110, were used for toxin production and were from the culture collection of the Department of Dairy and Food Industry, Iowa State University, Ames.

The organisms were grown and toxin was produced in cellophane dialysis bags immersed in culture medium as described by Boroff (1). This medium is similar to that described by Gordon et al. (6) for the production of toxin; it consists of 20 g of Proteose Peptone No. 1 (Difco), 20 g of yeast extract (Difco), and 10 g of glucose in 1,000 ml of distilled water. The *p*H of the medium was adjusted to 7.2 before sterilization.

The cellophane bag was filled with 0.85% saline solution before being suspended in the medium. The flask containing the cellophane bag was autoclaved for 15 min at 121 C. The rubber stopper was placed loosely in the neck of the flask during sterilization. After cooling, 1 ml of a culture of *C. botulinum*, which had previously been incubated in Cooked Meat Medium (Difco) for 24 hr, was inoculated into the bottom of the cellophane bag.

The inoculated material was incubated at 30 C for 7 to 14 days in an anaerobic incubator (National Appliance Co., Portland, Ore.). The chamber of the incubator was evacuated to 28 inches of vacuum with a vacuum pump and was flushed with nitrogen three times to obtain anaerobic conditions. After incubation, the contents of the dialysis bag were removed and centrifuged at 27,000 $\times$ *g* for 30 min at 4 C in a Sorvall RC2-B refrigerated centrifuge. The specificity of the gel-diffusion technique was determined with the toxic material produced by this culture method.

Mouse LD_{50} determinations. The biological activity of the toxins was determined by a mouse bioassay method similar to the procedure described by Schantz (10). Botulinal toxins types A, B, and E were diluted in 10-fold dilutions with 0.85% sterile saline. The dilutions ranged from 10^{-1} to 10^{-9}. A volume of 0.5 ml of each dilution of types A, B, and E toxins produced in the culture medium were inoculated intraperitoneally into each of 10 white mice each weighing 20 to 22 g. A control group of 10 white mice was inoculated with sterile saline only. Deaths were recorded at the end of 96 hr, and the LD_{50} values were calculated by the method of Reed and Muench (9).

Botulinal antitoxins. Types A, B, and E equine antitoxins were obtained from the Communicable Disease Center, Atlanta, Ga. When the antisera were rehydrated as directed on the bottles, they contained 10 International Units/ml as defined by Bowmer (2).

Gel-diffusion technique. The slide modification of the double gel diffusion, or Ouchterlony gel diffusion, was employed to detect botulinal toxin (5, 11).

Glass microscope slides (1 $\times$ 3 inches) were used for the gel-diffusion plates. The diffusion medium was 1% Special Nobel Agar (Difco) in borate saline

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buffer with 0.85% NaCl. The *p*H of the agar was adjusted to 7.8. Merthiolate was added to a final concentration of 1 part per 10,000 parts (total volume) to prevent bacterial growth. A volume of 4.5 ml of melted agar was pipetted onto the glass slide and allowed to harden. Wells were cut into the agar with the aid of a metal agar-cutter and plastic cutter-guide. The slide, shown in Fig. 1, has six wells, each 6 mm in diameter and evenly spaced around a 6-mm diameter center well. The distance between the center well and the six concentric wells is 5 mm.

Generally, the antisera were placed in the outer wells, and the toxic material was placed in the center well. Formation of a white precipitin line, as shown in Fig. 2 between the center well and one of the outer wells containing an antiserum, indicated a positive reaction.

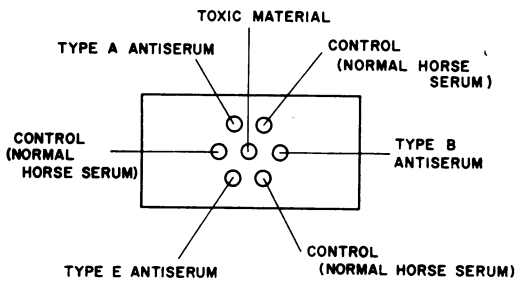

FIG. 1. *Drawing of Ouchterlony gel-diffusion plate with toxic material in center well and antisera in outer wells.*

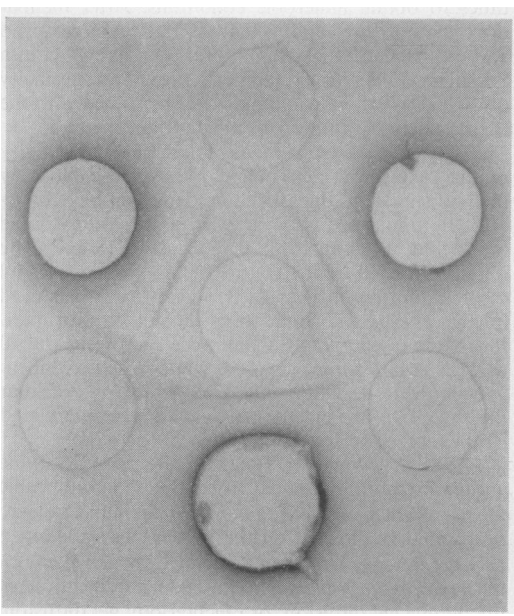

FIG. 2. *Gel-diffusion plate showing precipitin lines between toxic material from green beans in center well and type E antiserum in three outer wells. Remaining wells were not used in this picture.*

To determine the sensitivity of the method, 10-fold dilutions of purified, crystalline, type A toxin were placed in the outer wells, and the antitoxin was placed in the center well. The readable limit of the gel-diffusion technique was measured by observing the last dilution well at which a line was visible after 48 hr.

Recovery of botulinal toxin from food. Botulinal toxin was experimentally produced in canned green beans. Approximately 400 ml of the canned green beans was transferred to a 1,000-ml Erlenmeyer flask, the *p*H was adjusted to 7.2 with 1 N NaOH, and the flask was autoclaved for 15 min at 121 C. After cooling, the flask was inoculated with 1 ml of a culture of *C. botulinum* types A, B, or E, which had been incubated in Cooked Meat Medium for 24 hr. The food was incubated for 14 to 21 days in the anaerobic incubator. After incubation, the liquid portion of the food sample was drained off and centrifuged at 27,000 $\times$ *g* for 20 min at 4 C in a refrigerated centrifuge. This was done to remove food particles and lipid material. The supernatant fluid was decanted and filtered through a glass funnel packed with glass wool. A 5-ml volume of fluid was added to a 5-ml glass bottle used in the Hemmings' filter (Colab Laboratories, Inc., Chicago Heights, Ill.). The Hemmings' filter consists of two 5-ml glass bottles attached to a metal filter body containing a Sterimat filter pad. Dry, powdered Sephadex G 25 fine (Pharmacia, Uppsala, Sweden) was added to the liquid sample contained in the filter bottle, until a semisolid slurry was formed. The filter assembly, sample side up, was placed in a 50-ml plastic centrifuge tube (Sorvall) and centrifuged at 755 $\times$ *g* for 10 to 15 min at 4 C in a refrigerated centrifuge. The material that migrated through the filter was collected in the sample-collection bottle on the Hemmings' filter. The original volume of 5 ml was reduced to 1 to 2 ml. This procedure was performed by use of four Hemmings' filters from which approximately 5 ml of partially concentrated sample was collected. The procedure was then repeated with the partially concentrated sample, and its volume was reduced to 1 ml or less. Starting with a 20-ml liquid sample, a final volume of 1 ml was obtained in less than 1 hr. Theoretically, the amount of toxin contained in 20 ml of sample would be concentrated in 1 ml of sample.

The concentrated sample was placed in the center well of the microdiffusion plate. A volume of 0.1 ml of sample was required to fill the well. A volume of 0.1 ml of types A, B, and E antisera were placed in the outer wells. One of the outer wells contained normal horse serum as a control. The slide was incubated at 37 C in a humid chamber for 18 to 48 hr and examined under a fluorescent desk lamp against a black background for the presence of white precipitin lines.

Results of the toxin assay obtained from the gel-diffusion test were compared with results obtained from duplicate samples assayed by the standard mouse protection test.

RESULTS AND DISCUSSION

Gel-diffusion specificity and sensitivity. The botulinal toxin obtained in crystalline form and that produced in the dialysis bags were used to determine the specificity of the gel-diffusion test.

Toxin types A, B, and E all developed visible precipitin patterns in the gel-diffusion plate. Generally, the precipitin lines became visible in the gel when the slide had been incubated for 18 hr at 37 C in a humid chamber.

Some cross-reactions occurred between toxins and antitoxins in the gel-diffusion test. Type A antitoxin gave precipitin lines with type B toxin. Precipitin lines were seen between type B antitoxin and type A toxin. No cross-reactions were observed in the antigen-antibody reactions between types A and E, or B and E. Johnson et al. (7) reported that cross-reactions between toxins and antitoxins of types A and B occurred in passive hemagglutination and bentonite flocculation tests. Despite cross-reactions, types A and B could be distinguished from each other in the gel, and it was possible to type them by this procedure. The most distinct precipitin line formed in the gel between the toxin and its homologous antitoxin, and less distinct lines formed between the toxin and the cross-reacting antitoxin.

The sensitivity of the gel-diffusion test was calibrated with pure crystalline type A toxin and found to be in the range of 1 to 3 μg; toxin concentrations below this level were not detectable. Campbell et al. (3) reported that the sensitivity range for the gel-diffusion test is 1 to 3 μg of soluble-antigen or antibody.

Concentration of the toxin in Sephadex. The specific toxicity of botulinal toxin types A and B has been reported by Schantz (10) to be 3.8×10^{10} LD_{50} mouse units per g and for type E, 0.6×10^{10} LD_{50} mouse units per g. This means that, to kill 50% of the mice, an inoculum of 2.62×10^{-11} g per mouse of purified type A or B toxin is required, and 1.66×10^{-10} g of type E toxin is required. The gel-diffusion test used in the experiment required 10^{-6} to 3×10^{-6} g of toxin before visible precipitin lines formed in the gel. The mouse test was more sensitive than the gel-diffusion test by a factor of 10^4. To compensate for the low sensitivity of the gel-diffusion test compared with the mouse test, a concentration procedure was employed.

Sephadex G-25 fine will retain 2.5 g of water per g and will exclude molecules with a weight above 5,000; in this instance, botulinal toxin would be present in the excluded solution. By centrifuging the slurry of Sephadex and toxin-containing green-bean brine, the molecules heavier than 5,000 were forced to migrate from the Sephadex while water was held back. This was done by placing the slurry in the Hemmings' filter assembly and applying a force of $755 \times g$ with a Sorvall RC2-B refrigerated centrifuge.

Some toxicity was lost during concentration. When green-bean brine containing type E trypsin-activated toxin was reduced in volume from 20 ml to 1 ml and then rediluted with 0.85% saline to 20 ml, the toxic activity was reduced from 560 LD_{50} mouse units per ml to 206 LD_{50} mouse units per ml. When the 20-ml sample was reduced in volume to 1 ml, biological activity increased to 4,120 LD_{50} mouse units per ml. Thus, reducing type E toxic solution by a factor of 20 results in an eightfold increase in toxic activity per unit volume. Entrapment of some of the toxin in the Sephadex and Sterimat filter pad used in the Hemmings' filter may partly explain the reduction in toxicity. Also, there was the possibility that some of the toxin was denatured during concentration.

No precipitin lines were observed in the gel between these toxic food samples and antisera until the food samples were concentrated by this procedure. For example, no precipitin lines were seen in the gel between a food sample containing 560 LD_{50} mouse units of type E toxin and type E antitoxin before the sample was concentrated. When the sample was reduced in volume from 20 ml to 1 ml in Sephadex, a precipitin line was observed between it and type E antitoxin. After this sample was rediluted to 20 ml, no precipitin line was seen between it and type E antiserum.

Detection of botulinal toxin in food. Botulinal toxin types B and E were successfully detected in green beans with the gel-diffusion procedure. Type A toxin with an activity of 630 LD_{50} mouse units per ml was not detected because hemolyzed red blood cells in type A antitoxin caused a cloudy white area to form in the gel around the well in which the serum was placed. The cloudiness masked the presence of white precipitin lines which formed between the antigen and antibody wells. The cloudiness also interfered with the precipitin line formed between purified, crystalline, type A toxin and type A antitoxin.

An attempt was made to reduce the interference caused by hemolysis by precipitating the globulin portion of the antiserum with half-saturated ammonium sulfate. The precipitated globulin did not give distinct lines in the gel-diffusion plate with purified type A toxin. If the antiserum had not contained hemolyzed red blood cells, it probably would have given a positive reaction to type A toxin contained in the green-bean sample.

Botulinal toxin type B was detected in the green-bean brine at a level of 370 LD_{50} mouse units per ml of sample. The liquid sample was concentrated from 40 ml to 1 ml with Sephadex. Type E botulinal toxin, which had been activated with 1% trypsin, was detected at a level of 557 LD_{50} mouse units per ml of food sample. In this

instance, the green-bean brine was concentrated from 20 ml to 1 ml before analysis with gel diffusion.

Green-bean brine was selected for detection of botulinal toxins because it is relatively free of lipids, is not highly colored, and does not have a colloidal nature. Interference with diffusion of reactants or visibility of precipitin patterns in the gel-diffusion test was observed when one or more of these characteristics was present in a food sample. Additional preparatory procedures need to be developed to allow this kind of food sample to be analyzed directly by gel diffusion.

Perhaps lower concentrations of toxin can be detected by the gel-diffusion techniques, if antitoxin with higher biological activity is used. The antitoxins used in this work contained 10 International Units of antitoxin per ml; also, these antisera were intended for in vivo toxin assays and are not necessarily suited for in vitro assays. Concentrating the sample by a factor of 100:1 to 200:1 would also increase the sensitivity of the procedure.

Botulinal toxin can be rapidly concentrated by using Sephadex G-25 and the Hemmings' filter. A toxic food sample, such as green-bean brine, can be assayed directly by gel-diffusion methods without separation or purification of the toxin. The gel-diffusion method is still not as sensitive as the mouse test, but some refinements in the technique may overcome this disadvantage to some extent.

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