Serological Studies of Types A, B, and E Botulinal Toxins by Passive Hemagglutination and Bentonite Flocculation

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Abstract

JOHNSON, H. M. (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio), K. BRENNER, R. ANGELOTTI, AND H. E. HALL. Serological studies of types A, B, and E botulinal toxins by passive hemagglutination and bentonite flocculation. J. Bacteriol. 91:967–974. 1966.—Formalized sheep red blood cells (SRBC), sensitized with types A, B, and E botulinal toxoids and toxins by bis-diazotized benzidine (BDB), were tested against A, B, and E antitoxins prepared in horses and rabbits. Type B antitoxin cross-reacted with A toxoid SRBC, but the reciprocal cross-reaction was not observed. E toxin SRBC were specifically agglutinated by E antitoxin. Flocculation of antigen-sensitized bentonite particles was less sensitive in titration of antitoxin than hemagglutination. Also, reciprocal cross-reactions were observed between types A and B antitoxins. Cross-reactions in both serological tests were eliminated by titration of antitoxins in the presence of the heterologous antigens, with no inhibitory effect on the homologous antitoxins. Generally, equine antitoxins were less suitable for agglutinations, especially of antigen-sensitized bentonite particles. Types A, B, and E antitoxins were specifically inhibited by 43, 39, and 245 mouse LD₅₀ of their respective homologous toxins in the hemagglutination-inhibition test. A, B, and E antitoxins were specifically inhibited by 500, 950, and 1,500 mouse LD₅₀ of their respective homologous toxins in bentonite flocculation inhibitions. Formalized SRBC sensitized with rabbit types A and B antitoxins by BDB were respectively clumped by as little as 0.75 to 1.3 mouse LD₅₀ of A toxin and 2.3 LD₅₀ of B toxin, whereas bentonite particles sensitized by the same antitoxins were specifically clumped by 150 LD₅₀ of A toxin and 630 LD₅₀ of B toxin. E antitoxin sensitization of SRBC or bentonite particles was not successful. Evidence is presented that indicates that the serological procedures are applicable to the detection of botulinal toxins in food.

There are at least six types of antigenically distinct Clostridium botulinum toxins distinguishable by animal protection tests. These botulinal toxins are designated A, B, C, D, E, and F (7). The antigenic specificity of these toxins may not be as clear and distinct when in vitro serological tests are used. Studies by Lamanna and Lowenthal (11) have shown, for example, that cross-precipitation occurs between type A botulinal toxin and type B antitoxin. Also, antiserum produced to type A toxin neutralizes the hemagglutinin of type B toxin. Conversely, antiserum to type B toxin neutralizes the hemagglutinin of type A toxin. Reports by Rycaj (14), Yafayer and Chepeler (18), and Sinitsyn (15) indicate that passive hemagglutination of botulinal antitoxin-coated, tanned sheep red blood cells (SRBC) by toxins is as sensitive in detecting botulinal toxins as animal assay. Rycaj, however, worked only with type A toxin, whereas Yafayer and Chepeler, working with both A and B toxins, could not distinguish between them. Sinitsyn was able to distinguish between the two toxins, but the potencies of the toxins used in his studies were much lower than those usually found with botulinal cultures. None of these investigators used antigen-sensitized SRBC in their investigations. A recent review by Petty (12) includes references to the passive hemagglutination procedures just described.

The present study was initiated in an effort to
increase understanding of the in vitro serological properties of types A, B, and E botulinal toxins, and with the hope of development of an in vitro serological test for the specific detection of botulin toxins in cultures and contaminated foods. The serological tests used for these studies were hemagglutination and bentonite flocculation.

**MATERIALS AND METHODS**

*Reagents.* Trypsin used in these studies was Difco 1:250. Fisher reagents were used to prepare 0.15 M phosphate-buffered saline, pH 7.2 (PBS; 16). Fisher reagents were also used to prepare 0.15 M phosphate buffer, pH 7.3 (PB; 10). Bovine serum albumin fraction V powder (BSA) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Solutions of 1 and 0.25% were prepared in PBS. Bis-diazotized benzidine (BDB) was prepared according to the method of Gordon et al. (10).

*Sources of purified toxins and toxoids.* Purified crystalline type A botulin toxin was obtained from Edward J. Schantz, U.S. Army Biological Laboratories, Fort Detrick. Botulin toxoids types A, B, and E, in approximately 0.5% formalin, were supplied by Matteo Cardella, U.S. Army Biological Laboratories. Toxin and toxoids were exhaustively dialyzed against PBS before immunization of rabbits and use in serological tests.

*Preparation of partially purified type E toxin for sensitization of erythrocytes and bentonite particles.* The VH strain of *C. botulinum* type E was grown in a cellophane bag that was constructed as described by Boroff (1). The toxin-production medium was prepared as described by Gordon et al. (9). The inoculum, 2 ml of an 18-hr culture, was added to 100 ml of physiological saline in the cellophane bag. The culture was incubated at 30 C for 7 days, after which the cellophane bag was removed from the medium and the contents were centrifuged at 15,000 rev/min for 1 hr in the International BD-2-ultracentrifuge (970 rotor). Six volumes of a solution of saturated (NH₄)₂SO₄ were added to 4 volumes of toxic supernatant fluid. After the toxin had been allowed to precipitate overnight at 4 C, it was collected by centrifugation at 15,000 rev/min for 1 hr in the BD-2 ultracentrifuge, dissolved in 10 ml of physiological saline and exhaustively dialyzed against saline, then recenterfuged at 15,000 rev/min for 1 hr. The supernatant liquid was removed, assayed for toxicity, and used for sensitization of erythrocytes and bentonite particles. The specific toxicity of the preparation was 6 X 10⁸ mouse LD₅₀ per mg of protein.

*Toxic cultures.* Types A (Hall strain), B, and E (VH strain) botulin cultures were supplied by Matteo Cardella, U.S. Army Biological Laboratories. The organisms were cultured for toxin production as described by Duff et al. (8) and Gordon et al. (9). Types A and B botulin toxins were acid-precipitated from cultures by adjustment of the medium to pH 3.2 with 1 N HCl, and were washed once with distilled water. Type E toxin was precipitated by mixing 6 volumes of a solution of saturated (NH₄)₂SO₄ with 4 volumes of the supernatant fluid of the centrifuged culture. The precipitated toxins were dissolved in PBS to one-tenth the original volume, exhaustively dialyzed against PBS, and centrifuged at 15,000 rev/min for 1 hr in the International BD-2-ultracentrifuge (969 rotor). The supernatant fluids were then assayed for toxicity and employed in inhibition of antitoxins and in agglutinins of antitoxin-sensitized erythrocytes and bentonite particles.

Cultures other than *C. botulinum* were from the stock culture collection of the Food Microbiology section of Milk and Food Research, Robert A. Taft Sanitary Engineering Center.

*Mouse LD₅₀ determinations of botulin toxins.* Types A and B botulin toxins were diluted in phosphate-buffered gelatin (pH 6.5). The buffer-gelatin solution was prepared as described by Crisley (6). Six mice, weighing 20 g each, were injected intraperitoneally with 0.5 ml of each dilution of toxin. Deaths were recorded at the end of 4 days, and LD₅₀ values were determined by the method of Reed and Muench (13). Type E toxin was diluted 1:10 in 0.1 M phosphate buffer (pH 6.5) containing a final concentration of 0.1% trypsin. The toxin and trypsin were incubated at 37 C for 60 min, after which the trypsin-treated toxin was dissolved in phosphate-buffered gelatin (pH 6.5) and injected into mice.

*Botulinal antitoxins.* Types A, B, and E equine antitoxins were obtained from the Communicable Disease Center (CDC), Atlanta, Ga. The stock antitoxins contained 10 international units (IU) of antitoxin per ml (2). Type E equine antitoxin was also obtained from the Microbiological Research Establishment, Porton, England. The Porton antitoxin contained 1,500 IU of antitoxin per ml.

Types A, B, and E antitoxins were also produced in rabbits by repeated intraperitoneal injections of A, B, and E botulin toxins in complete Freund's adjuvant. Two rabbits were injected with 2 mg of each toxin bimonthly. The rabbits were bled 3 weeks after the final injection by cardiac puncture. Samples of the sera were stored at - 20 C. Prior to use in serological tests, the sera were inactivated at 56 C for 30 min. Before use in hemagglutination procedures, all sera were absorbed with equal volumes of packed SRBC. Antiserum to the individual toxoids had similar potency, whereas that of the toxin was available; therefore, the potency of the E antitoxin produced in one rabbit was found to be 18 IU per ml, with Porton equine antitoxin as reference (2). Standard antitoxins to A and B toxoids were not available; therefore, the potencies of rabbit types A and B antitoxins were not expressed in terms of international units.

*Formaldehyde treatment of erythrocytes.* The method of Butler (5) was employed for formalization of SRBC. The formalized SRBC were stored at 4 C as a 50% suspension in PBS. The final cell suspension contained 0.01% thimerosal.

*Coupling of antigens to formalinized SRBC by BDB.* Toxoids and toxins were coupled to SRBC by modification of the procedure described by Butler (5). A 0.1 ml amount of a 50% suspension of formalized SRBC was added to toxoid (or toxin) contained in 3 ml of 0.15 M PB. BDB was thawed, diluted 1:15 with PB, and 0.75 ml of the diluted BDB was added to the
mixture of toxoid and SRBC. The mixture was incubated at room temperature for 12 min, followed by centrifugation of the mixture at 2,500 rev/min for several minutes to sediment the cells. The cells were washed twice with PB and resuspended to 2.5 ml with 0.25% BSA. Thimerosal was added to a final concentration of 0.01% if the cells were not used immediately. The optimal concentrations of types A and B toxoids for sensitization of cells were, respectively, 100 and 200 μg of toxoid per ml. Partially purified type E toxoid at a concentration of 100,000 LD₅₀ per ml was used for sensitization of formalized SRBC for hemagglutination by E antitoxin. Type E toxoid was not used for sensitization of SRBC because of contamination with trypsin. The toxoid was prepared according to the method of Gordon et al. (9), which included trypsin activation in the purification procedure. Rabbits immunized with the toxoid produced, after absorption of antibodies to the trypsin, which masked the toxoid-antitoxin reaction. The toxins and toxoids used for sensitization of cells were devoid of hemagglutinating activity at concentrations used.

Hemagglutination of antigen-sensitized formalized SRBC by antitoxin. Hemagglutinations were carried out by serial twofold dilution of antiserum in 0.5-ml volumes with 0.25% BSA. To each dilution of antiserum was added 0.05 ml of antigen-sensitized formalized SRBC. The mixture was shaken, incubated at room temperature for 4 to 18 hr, and then read for agglutination by pattern formation. The titer was considered as the highest dilution of antitoxin that produced unequivocal clumping of the cells.

Inhibition tests. Hemagglutination-inhibition tests were performed by mixing various concentrations of toxins from toxic cultures in 0.25-ml quantities in 0.25% BSA with 0.25 ml of antitoxin that had been diluted with 0.25% BSA to contain a three-plus hemagglutinating dose of antitoxin. After the mixtures had incubated for 20 min at room temperature, antigen-sensitized formalized SRBC were added. The contents were mixed, incubated at room temperature for 4 to 18 hr, and read for pattern development. Preparation of antitoxin-sensitized formalized SRBC. To 1 ml of rabbit antitoxin that had been diluted to 10 ml with distilled water, was added 5 ml of a saturated solution of (NH₄)₂SO₄. The mixture was allowed to stand for 15 min, after which it was dissolved in 5 ml of PBS and dialyzed against several changes of PBS. The dialyzed antitoxin globulin was then cleared of particulate matter by centrifugation at 15,000 rev/min for 30 min. The cleared antitoxin globulin was measured in a Beckman DU spectrophotometer at a wavelength of 278 μm. The antitoxin globulin was then added to 1.5 ml of diluted DBB as described for antigen coupling. The antitoxin-sensitized cells were suspended in 2.5 ml of 0.25% BSA.

Hemagglutination of antitoxin-sensitized formalized SRBC by botulinum toxins. Crystalline type A botulinum toxin and botulinum toxins removed from cultures by acid or (NH₄)₂SO₄ precipitation were serially diluted in 0.5-ml volumes in 0.25% BSA. To each dilution of toxoid was added 0.05 ml of antitoxin-sensitized formalinized SRBC. The tubes were incubated and the test was read as described for hemagglutination of antigen-sensitized cells by antitoxin. Controls consisted of a tube of the highest concentration of toxoid in the test with untreated formalized SRBC (2.5% solution) and a tube containing antitoxin-sensitized formalinized SRBC and 0.25% BSA.

Flocculation of antigen-sensitized bentonite particles. Stock bentonite (Vocolay, American Colloid Co., Skokie, Ill.) was prepared by the method of Bozicevich et al. (3). Bentonite particles were sensitized with types A and B botulinum toxoids and partially purified type E toxoid by a modification of the method of Wolff et al. (17). To 10 ml of stock bentonite suspension that had been concentrated to 1 ml in distilled water by centrifugation was added 1 ml of toxoid or toxin containing 500 to 1,000 μg of types A and B toxoid and 100,000 LD₅₀ of E toxoid. After incubation of bentonite and antigen for 1 hr at room temperature, 1 ml of 1% BSA, 15 ml of distilled water, and 0.1 ml of 1% methylene blue were added. The antigen-sensitized bentonite particles were centrifuged and washed twice with 1 ml of 1% BSA and 15 ml of distilled water. The bentonite particles were suspended in 1% BSA to a final concentration of 150,000 particles per mm³. For the test, serum was serially diluted in 0.1-ml volumes in PBS on microfloculation slides. To each dilution of serum was added 0.05 ml of antigen-sensitized bentonite. The mixture was rotated at 120 rev/min on a variable-speed rotator (Yankee-type from Clay-Adams Inc., New York, N.Y.) for 20 min, after which the antigen-coupled bentonite particles were examined for clumping under a microscope at 100 times magnification.

Bentonite flocculation inhibition. Bentonite flocculations were performed by serial dilution of the acid- and (NH₄)₂SO₄-precipitated toxins in 0.1-ml volumes in tubes (10 by 75 mm). To each dilution of toxoid was added 0.1 ml of antitoxin. The antitoxin had been diluted to contain a three-plus agglutinating dose of antitoxin in 0.1 ml after having been mixed with the toxoid. After incubating at room temperature for 20 min, 0.1 ml of each dilution of the mixture of inhibitor and antitoxin was added to microfloculation slides, and bentonite flocculation was carried out as described above.

Flocculation of antibody-sensitized bentonite particles. Bentonite particles were sensitized with varying amounts of (NH₄)₂SO₄-precipitated antitoxins according to the method of Bozicevich et al. (4), except that 1% BSA was used as the stabilizing agent. Toxins were diluted in 0.1-ml volumes. To each dilution of toxoid was added 0.5 ml of antibody-sensitized bentonite particles, and bentonite flocculations were carried out.

Preliminary tests on culture supernatant fluids and foods for botulinum toxins. Supernatant fluids from cultures of C. botulinum types A, B, and E, acid precipitates of canned corn and beans that were inoculated with C. botulinum type A, along with a specimen of food that had been implicated in a case of C. botulinum type B intoxication, were examined for...
botulinal toxins by some of the serological techniques described.

RESULTS

Data on passive hemagglutination and bentonite flocculation with types A and B toxins and type E toxin, and their corresponding antitoxins, are presented in Table 1. Types A and B antitoxins are of equine origin (CDC). The E antitoxin is of rabbit origin. A strong cross-reaction occurred between type B antitoxin and A toxoid SRBC, whereas A antitoxin did not react with B toxoid-sensitized SRBC. Neither A nor B antitoxins cross-reacted with E toxin-sensitized SRBC. Similarly, E antitoxin did not cross-react with A or B toxoid SRBC. A pattern of reactivity similar to that of passive hemagglutination was also observed in bentonite flocculations, except that A antitoxin strongly cross-reacted with B toxoid bentonite particles. The sensitivities of the homologous bentonite flocculations for A, B, and E antitoxins were, respectively, 21-, 4.7-, and 4.5-fold less than in passive hemagglutination. Both CDC and Porton type E equine antitoxins failed to clump E toxin bentonite particles. CDC equine antitoxin also failed to agglutinate E toxin SRBC, but the Porton anti-E reacted with E toxin SRBC to a titer of 0.0368 IU of antitoxin (1:40,960 dilution) per ml. In both the hemagglutination and bentonite flocculation tests, the cross-reactions were eliminated by titration of antitoxins in the presence of the cross-reacting antigens, with no reduction in titer of the homologous reactions. For this, 20 μg of the appropriate A or B toxoid, contained in 0.1 ml, was added to each dilution of antitoxin. The two were mixed and incubated at room temperature for 20 min, followed by the addition of antigen-sensitized SRBC or bentonite particles to each tube and the performance of the passive agglutination test.

Results of hemagglutination and bentonite flocculation of A and B toxoid-sensitized SRBC and A and B toxoid-sensitized bentonite particles by rabbit types A and B antitoxins are presented in Table 2. The reactions were similar to those of equine antitoxins (Table 1), except that titers were considerably higher than with the equine sera. Type A antitoxin did not cross-react with B toxoid SRBC, but did agglutinate B toxoid bentonite particles. Rabbit B antitoxin crossed-reacted with A toxoid in both hemagglutinations and bentonite flocculations.

Data on hemagglutination inhibition with types A, B, and E rabbit antitoxins by A, B, and E toxic cultures are presented in Table 3. The rabbit antitoxins are the same as those used in the hemagglutinations reported in Tables 1 and 2. The acid- and (NH₄)₂SO₄-precipitated toxins were employed against a three-plus hemagglutinating dose of antitoxin. Types A, B, and E antitoxins were specifically inhibited by 43, 39, and 245 mouse LD₅₀ of the respective toxins. Similar results were obtained when equine antitoxins were used in the inhibitions. Results of bentonite flocculation inhibitions with types A, B, and E antitoxins by A, B, and E toxic cultures are summarized in Table 4. Antitoxins that were employed in the bentonite flocculations of Table 1 were used. Although the inhibitions were specific, they were not as sensitive as the hemagglutination inhibitions. Prior treatment of type E toxin with a final concentration of 0.1% trypsin did not enhance its

| Table 1. Passive hemagglutination and bentonite flocculation with types A, B, and E botulinal toxoids and their corresponding antitoxins |
|---|---|---|
| Serological test | Toxoid | Antitoxin (international units per ml)* |
| | | A | B | E |
| Passive hemagglutination | A | 0.0007 (12800) | 0.0125 (800) | >0.18 (100) |
| | B | >0.1 (100) | 0.0015 (6400) | >0.18 (100) |
| | E | >0.1 (100) | >0.1 (100) | 0.002 (8192) |
| Bentonite flocculation | A | 0.015 (640) | 0.03 (320) | >9 (2) |
| | B | 0.06 (160) | 0.007 (1280) | >9 (2) |
| | E | >1 (10) | >1 (10) | 0.009 (2048) |

* The values denote smallest number of international units of antitoxin per milliliter that produced unequivocal clumping of the antigen-sensitized SRBC and bentonite particles; >0.1, >0.18, >1.0, and >9 indicate a negative reaction with the highest concentration of antitoxin used. Values in parentheses indicate reciprocal of serum dilution.

* Equine antitoxins from Communicable Disease Center.
* Anti-E produced in rabbits.
* Partially purified E toxin.
TABLE 2. Passive hemagglutination and bentonite flocculation with types A and B rabbit botulinal antitoxins

| Serological test          | Type A | Type B | Passive hemagglutination
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>Reciprocal of dilution</td>
</tr>
<tr>
<td>Passive hemagglutination</td>
<td>20,900,000</td>
<td>650,000</td>
<td></td>
</tr>
<tr>
<td>Bentonite flocculation</td>
<td>204,800</td>
<td>25,600</td>
<td></td>
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</tbody>
</table>

* Indicates negative reaction at 1:1,000 dilution.

TABLE 3. Hemagglutination inhibition with types A, B, and E botulinal antitoxins

| Antitoxin* | Inhibitor | Amount of inhibitor (mouse LD<sub>50</sub>)<sup>a</sup> | Passive hemagglutination
<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Type A</td>
<td>A toxin</td>
<td>43</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>B toxin</td>
<td>2,450</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>E toxin</td>
<td>1,570</td>
<td>3</td>
</tr>
<tr>
<td>Type B</td>
<td>A toxin</td>
<td>5,500</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B toxin</td>
<td>39</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E toxin</td>
<td>1,570</td>
<td>3</td>
</tr>
<tr>
<td>Type E</td>
<td>A toxin</td>
<td>55,000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B toxin</td>
<td>2,450</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>E toxin</td>
<td>245</td>
<td>—</td>
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</table>

* Antitoxins produced in rabbits.

* Values represent the smallest amount of toxin (in 0.25 ml) in terms of LD<sub>50</sub> that completely inhibited a three-plus agglutinating dose of antitoxin or the largest amount of toxin that had no effect on antitoxin.

* Hemagglutination patterns were recorded as described by Butler (5). This scheme of notation also holds for hemagglutination recordings in Tables 3 and 7.

inhibitory properties in either the hemagglutination or bentonite flocculation procedures.

The toxins that were used for inhibitions in Tables 3 and 4 were also used for hemagglutination of types A and B antitoxin-sensitized SRBC (Table 5). The rabbit antitoxins that were employed in the hemagglutinations and bentonite flocculations summarized in Table 2 were also employed for sensitization of formalinized SRBC. Equine antitoxins from the CDC were unsuitable for antibody sensitization of SRBC. SRBC were sensitized with 100 and 200 µg of antitoxin globulin per ml. Type A botulinal toxin was detected in quantities as low as 1.3 mouse LD<sub>50</sub> per 0.5 ml by both 100 and 200 µg of A antitoxin SRBC. A antitoxin SRBC did not cross-react with B toxin. Type B botulinal toxin was detected in quantities as low as 9 LD<sub>50</sub> per 0.5 ml for 100 µg of B antitoxin SRBC and 2.3 mouse LD<sub>50</sub> for 200 µg of B antitoxin SRBC. Strong cross-reactions of A toxin with B antitoxin SRBC also occurred. Results of similar studies with antibody-sensitized bentonite particles are presented in Table 6. The specificity of the reactions depended on the amount of antitoxin globulin used for sensitization of the bentonite. For example, strong cross-reactions occurred in both systems when 5,000 µg of antitoxin was used for sensitization of the bentonite particles. When 500 µg of antitoxin globulin was used for sensitization, type A toxin could be specifically detected in quantities as low as 140 mouse LD<sub>50</sub> per 0.1 ml and type B toxin at 630 LD<sub>50</sub> per 0.1 ml. Supernatant fluids from cultures of an enterococcus, Micrococcus, and Bacillus species, and from one strain each of C. botulinum type E, C. perfringens, C. sporogenes, C. bifermentans, C. butyricum, C. multifermentans, C. putrefaciens, and C. tetani did not clump either A antitoxin- or B antitoxin-sensitized SRBC or bentonite particles. The antitoxin-sensitized SRBC and bentonite techniques were not success-

TABLE 4. Bentonite flocculation inhibition with types A, B, and E botulinal antitoxins

| Antitoxin* | Inhibitor | Amount of inhibitor, mouse LD<sub>50</sub> | Bentonite flocculation
<table>
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<tbody>
<tr>
<td>Type A</td>
<td>A toxin</td>
<td>1,000</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>B toxin</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E toxin</td>
<td>6.3 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>Type B</td>
<td>A toxin</td>
<td>1.4 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B toxin</td>
<td>1,900</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>E toxin</td>
<td>9.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Type E</td>
<td>A toxin</td>
<td>1.4 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B toxin</td>
<td>6.3 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>E toxin</td>
<td>1,500</td>
<td>—</td>
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* Types A and B antitoxins are of equine origin and were obtained from the CDC. Type E was produced in rabbits.

* Values represent the smallest amount of toxin (in 0.1 ml) in terms of LD<sub>50</sub> that completely or partially inhibited a three-plus agglutinating dose of antitoxin or the largest amount of toxin used that had no effect on antitoxin.

* Bentonite flocculations were recorded as described by Bozicevich et al. (3). This scheme of notation also holds for flocculation recordings in Table 6.
ful with E antitoxin from either rabbit or equine sources.

Data on the sensitivity of the antitoxin SRBC technique in detecting A toxin in a preparation of crystalline type A botulinal toxin is presented in Table 7. The test could detect as little as 0.75 mouse LD$_{50}$ of toxin in 0.5 ml, corresponding to 0.000058 $\mu$g of toxin. Hemagglutination inhibition detected a minimum of 50 LD$_{50}$ of toxin, corresponding to 0.00375 $\mu$g.

Preliminary studies with the serological techniques just described indicate that A, B, and E toxins can be specifically detected in supernatant fluids from types A, B, and E C. botulinum cultures. Toxins acid-precipitated from canned corn and beans that were inoculated with a C. botulinum type A culture, as well as from an outbreak food (home-canned fruit obtained from the CDC) containing type B toxin, also gave specific positive results when tested by hemagglutination inhibition and hemagglutination of antitoxin-sensitized SRBC. The sensitivity of all of these tests was comparable to that obtained with toxins acid- and (NH$_4$)$_2$SO$_4$-precipitated from cultures. In addition, tests on acid precipitates of a variety of foods not contaminated with botulinal toxins indicate that the food components do not interfere with the serological techniques. Detailed studies are in progress to determine further the applicability of these serological techniques for the detection of botulinal toxins in foods.

**DISCUSSION**

The BDB hemagglutination procedure was considerably more sensitive in titration of antitoxins than was bentonite flocculation. Also, surprisingly, cross-reactions were less in the hemagglutination test, since no cross-reactions occurred between type A equine or rabbit antitoxin and B toxoid SRBC, whereas strong cross-reactions occurred in the bentonite flocculation procedure. Cross-reaction of type B antitoxin with A toxoid occurred in both tests. Perhaps B toxoid is attached to SRBC by BDB in a manner that ster-

<table>
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<th>TABLE 5. Passive hemagglutination of antibody-sensitized formalinized SRBC by types A and B botulinal toxins</th>
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<tbody>
<tr>
<td><strong>Toxin (LD$_{50}$ per 0.5 ml)</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>A ($7 \times 10^6$)</td>
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<td></td>
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<tr>
<td>B ($3.1 \times 10^6$)</td>
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<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 6. Bentonite flocculation of antibody-sensitized particles by types A and B botulinal toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toxin (LD$_{50}$ per 0.1 ml)</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>A ($1.4 \times 10^4$)</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>B ($6.3 \times 10^4$)</td>
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</tr>
</tbody>
</table>
## Table 7. Passive hemagglutination of A antitoxin-sensitized formalized SRBC by crystalline type A botulinic toxin

<table>
<thead>
<tr>
<th>Mouse LD₅₀ of toxin per 0.5 ml</th>
<th>Amt (µg) of toxin per 0.5 ml</th>
<th>SDB hemagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>0.015</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>0.0075</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>0.00375</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>0.00188</td>
<td>3</td>
</tr>
<tr>
<td>12.5</td>
<td>0.00094</td>
<td>3</td>
</tr>
<tr>
<td>6.25</td>
<td>0.00047</td>
<td>3</td>
</tr>
<tr>
<td>3.125</td>
<td>0.000235</td>
<td>3</td>
</tr>
<tr>
<td>1.5</td>
<td>0.000117</td>
<td>2</td>
</tr>
<tr>
<td>0.75</td>
<td>0.000059</td>
<td>1</td>
</tr>
<tr>
<td>0.375</td>
<td>0.000029</td>
<td>-</td>
</tr>
<tr>
<td>Toxin control*</td>
<td>0.015</td>
<td>-</td>
</tr>
</tbody>
</table>

* Untreated formalized SRBC were used.

cally blocks the heterologous reaction. All of these cross-reactions were eliminated by titration of antitoxins in the presence of the heterologous toxoids, with no reduction in titers of the homologous antitoxins. The nature of the moiety of the toxins that is responsible for the cross-reactions is not known with certainty, but previous studies by Lamanna and Lowenthal (11), along with preliminary data obtained in our laboratory by partial separation of the toxic moiety of type A from the hemagglutinin by density gradient ultracentrifugation, suggest that the hemagglutinin of the toxins is involved. Apparently the hemagglutinin is serologically active at dilutions in which hemagglutination does not occur.

The use of formaldehyde-treated SRBC provides a stable hemagglutination system. Also, as shown by Lamanna and Aragon (Bacteriol. Proc., p. 94, 1956), formaldehyde destroys red blood cell receptors for the botulinic hemagglutinin, thus making it unlikely that the hemagglutinin of the toxin and toxoid can of itself cause hemagglutination of formaldehyde-treated SRBC.

Hemagglutination inhibition and bentonite flocculation inhibition appear to be quite suitable for the rapid and specific detection of botulinic toxins types A, B, and E in cultures and foods. Hemagglutination inhibition detects toxin at levels well below that which is fatal to human beings.

The antitoxin-sensitized SRBC technique is extremely sensitive, detecting toxins at levels of less than one to several LD₅₀ per 0.5 ml. The strong cross-reaction of B antitoxin-SRBC with A toxin does not hinder the differential diagnostic potential of this test, since no cross-reaction occurred between A antitoxin SRBC and type B botulinic toxin. With the use of the same antitoxins, anti-toxin-bentonite flocculation was considerably less sensitive than hemagglutination, detecting 140 mouse LD₅₀ per 0.1 ml of A toxin and 630 LD₅₀ of B toxin. The potency of CDC equine antitoxins, in terms of serum dilutions, was considerably less than that of rabbit antitoxins types A and B. This lower potency may account for the inability of CDC equine antitoxins to sensitize SRBC and bentonite particles. The inability of rabbit type E antitoxin to work in the antitoxin SRBC test may also be due to low antibody content of the serum. The hemagglutination titer of rabbit type E antitoxin was considerably lower than that of rabbit types A and B antitoxins.

The diagnostic equine antitoxins presently available to botulinic toxins may not be the most suitable for in vitro serological studies of botulinic toxins. This observation is based on the unsuitability of equine types A and B antitoxins (CDC) to sensitize SRBC and bentonite particles and the inability of equine type E antitoxin (CDC) to clump either antigen-sensitized SRBC or bentonite particles.

### Literature Cited

9. **Gordon, M., M. A. Flock, A. Yarinsky, and J. T. Duff.** 1957. Studies on immunity to toxins of *Clostridium botulinum*. III. Preparation,
purification, and detoxification of type E toxin.


