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Isolation and Some Properties of an Enterotoxin Produced by *Bacillus cereus*

N. E. THOMPSON, M. J. KETTERHAGEN, M. S. BERGDOLL, AND E. J. SCHANTZ*
Food Research Institute, University of Wisconsin-Madison, Madison, Wisconsin 53706

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Extracellular proteins produced by *Bacillus cereus* B-4ac were separated by chromatography on Amberlite CG-400, QAE-Sephadex, Sephadex G-75, and hydroxylapatite. A fraction, containing three detectable antigens, obtained from chromatography on hydroxylapatite caused fluid accumulation in ligated rabbit ileal loops, was dermonecrotic to rabbit skin, was cytotoxic to cultured cells, and was lethal to mice after intravenous injection. Two other fractions obtained from chromatography on hydroxylapatite showed essentially no toxic activity when tested individually. Each nontoxic fraction contained two of the three proteins present in the toxic material. When the two nontoxic fractions were combined, activity in all of the biological assays was observed. Antiserum against either of the nontoxic fractions neutralized the dermonecrotic response of the combined material. These results suggest that all of these biological activities probably are due to a single entity and that more than one component probably comprise the toxic entity.

The association of *Bacillus cereus* with food-borne illness is now well established and has been reviewed recently (10, 11, 23). Two types of food-borne illness are recognized, one causing diarrhea and the other causing emesis. The ability of *B. cereus* to cause diarrhea has been attributed to the production of a proteinaceous enterotoxin (12, 13, 20, 22). This study is concerned with a toxin produced by *B. cereus* B-4ac that is believed to be the agent responsible for the induction of diarrhea.

Like culture filtrates of *Vibrio cholerae* (4, 5), enterotoxigenic *Escherichia coli* (7, 17), and cell extracts of *Clostridium perfringens* (6, 18), culture filtrates of *B. cereus* cause fluid accumulation in rabbit ileal loop (RIL) assays (21) and increase vascular permeability (VP) in rabbit skin (12). The VP activity of *B. cereus* culture filtrates correlates well with RIL activity (12, 24). Other toxic effects of *B. cereus* culture filtrates include cytotoxicity to cultured cells (1) and lethality to mice after intravenous injection (3). However, attempts to isolate the factor(s) responsible for these biological activities and to relate them to diarrheal activity have resulted in inconclusive findings.

Attempts by Spira and Goepfert (21) to isolate the factor responsible for the VP activity resulted in separation of VP activity from hemolytic and phospholipase activities by ammonium sulfate precipitation of crude culture supernatant fluids and subsequent chromatography on Sephadex G-75. Unable to duplicate this separation procedure, Turnbull et al. (24) used preparative isoelectric focusing to separate proteins in crude culture supernatant fluids. They were able to isolate a fraction enriched for VP and RIL activity and devoid of phospholipase activity, but this fraction contained residual hemolytic activity. Ezepchuk et al. (8) reported the isolation of a permeability factor of molecular weight 100,000; this factor was lethal to mice, but did not give an RIL response.

The resolution of the various biological manifestations of *B. cereus* metabolites has been impeded by the absence of a reproducible, large-scale purification procedure for the toxic factor(s). We report here the isolation of two fractions by chromatographic procedures that were devoid of biological activity when assayed individually, but demonstrated activity in the RIL and VP tests when combined. This reconstructed toxic material was also cytotoxic to cultured cells and lethal to mice after intravenous injection.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strain and toxin production. *B. cereus* strain B-4ac, originally isolated from a food poisoning outbreak, was used throughout this study and has been described previously (13, 21, 22, 24). Stock cultures were maintained on nutrient agar (Difco Laboratories, Detroit, Mich.) slants at 4°C. For toxin production, a fresh slant culture, incubated overnight at 30°C, was used to inoculate two 250-ml Erlenmeyer flasks each containing 100 ml of brain heart infusion (BHI) broth (Difco). After shaking (280 rpm) for at least 10 h at 35°C, the cultures were combined, and a 5-ml volume was transferred to each of 24 500-ml volumes of BHI contained in 2-liter Erlenmeyer flasks. These cultures were incubated with shaking (140 rpm) for 8 to 10 h at 30°C. Bacterial cells were removed by centrifugation (25,000 × *g*, 30 min).

Isolation of the toxic factor. Figure 1 is a schematic representation of the isolation procedure. Details of this procedure are described below.

The culture supernatant from 12 liters of culture was dialyzed against 110 liters of phosphate buffer (0.02 M, pH 6.0) containing 0.02% NaN_3 for 18 h at 4°C. This material was then stirred with approximately 400 ml of preswollen, precycled (acid-base) Amberlite CG-400 (Sigma Chemical Co., St. Louis, Mo.) for 2 h at room temperature.

The fluid was decanted from the resin and adjusted to pH 7.0 by the slow addition of 5 N NaOH. Approximately 600 ml of preswollen QAE-Sephadex (A50) (Pharmacia Fine Chemicals, Piscataway, N.J.) that had been equilibrated in phosphate buffer (0.02 M, pH 7.0) was added and stirred for 18 h at 4°C. The QAE gel, containing the toxic factor, was packed into a column, washed with 2 liters of phosphate buffer (0.02 M, pH 7.0), and then eluted sequentially with this buffer containing 0.05 M NaCl and 0.10 M NaCl (approximately 2 liters of each). The material eluted with the 0.10 M NaCl, containing the toxic factor, was designated QAE(0.1) and concentrated by dialysis against a slurry of polyethylene glycol (20 M; Union Carbide Corp., Danbury, Conn.) in phosphate buffer (0.02 M, pH 8.0) to approximately 15 ml.

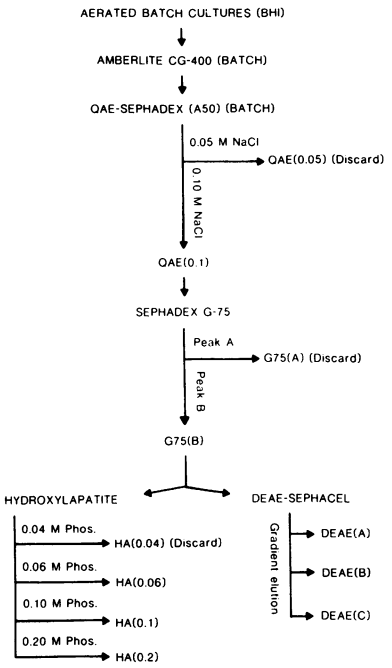

FIG. 1. Schematic representation of the procedure used to isolate the toxic HA(0.1) material and the antigens that comprised this material.

The concentrated QAE(0.1) material was applied to a column (4 by 100 cm) of Sephadex G-75 (Pharmacia) in phosphate buffer (0.02 M, pH 8.0) containing 0.15 M NaCl. The column was developed at a flow rate of 25 ml/h at 4°C. Fractions constituting the second peak (Fig. 2) were pooled and designated G75(B).

A portion of the G75(B) material was dialyzed against phosphate buffer (0.01 M, pH 7.0) and applied to a column (1.1 by 8.0 cm) of Hydroxylapatite Fast Flow (Calbiochem, La Jolla, Calif.) equilibrated in the sample buffer. The adsorbed substances were eluted stepwise with 0.04, 0.06, 0.10, and 0.20 M phosphate buffer (pH 7.0) at room temperature.

Another portion of the G75(B) material was dialyzed against phosphate buffer (0.02 M, pH 8.0) and applied to a column (0.9 by 11.0 cm) of DEAE-Sephacel (Pharmacia) equilibrated in the same buffer. After washing with the starting buffer, the adsorbed substances were eluted with a gradient ranging from 0 NaCl (400 ml) to 0.175 M NaCl (400 ml) in the starting buffer at a flow rate of about 20 ml/h at 4°C.

Biological assays. RIL assays were performed by the procedure of Spira and Goepfert (21) with several modifications. Female New Zealand White rabbits weighing 700 to 1,000 g were anesthetized with Halothane (Halocarbon Laboratories, Inc., Hackensack, N.J.). Ligations were made at least 6 cm from the appendix and spaced to yield up to seven test loops (averaging 6 cm in length) per animal; the test loops were separated by interloops of approximately 4 cm in length. All samples were dialyzed against phosphate buffer (0.02 M, pH 7.0) and sterilized by passage through a 0.2-μm, Acrodisc (Gelman Sciences, Inc., Ann Arbor, Mich.) before use. Each animal received up to seven 2.0-ml samples, including a positive control (crude culture supernatant concentrated about 20 times) and a negative control (phosphate buffer). Each set of samples was tested in at least seven rabbits, and the position of each sample was randomized for each animal. Animals were held for 5 h postsurgery before they were sacrificed. Loops containing fluid were aspirated to measure the fluid and then measured to determine the exact length. A positive response was defined as a volume/length ratio greater than or equal to 0.50 in at least 50% of the test animals. Animals that responded to the negative control, did not respond to the positive control, or showed a positive response in any interloops were discarded from the test.

VP activity was determined by the method of Glatz et al. (12). Each sample was dialyzed against phosphate buffer (0.02 M, pH 7.0) and injected (0.05 ml) in duplicate into at least two 2-kg female New Zealand White rabbits. After 3 h each animal was injected with 2 ml of a 10% solution of Evans blue (Fisher Scientific Co., Fair Lawn, N.J.) prepared in 0.9% NaCl; reactions were read after an additional 1 h. Activity was characterized as either bluing or necrosis and expressed as the reciprocal of the dilution resulting in a zone of less than 1.0 mm in diameter.

Monkey feeding trials were performed by the method of Goepfert (13). All feedings were performed at the Wisconsin Regional Primate Research Center at the University of Wisconsin, with Rhesus monkeys of either sex weighing 1.5 to 3.0 kg. Four to eight animals were used per trial and were observed for 6 h for passage of soft stools.

Cytotoxic activity was determined by the use of Vero cells obtained from J. Stelma (U.S. Food and Drug Administration; Cincinnati, Ohio). Cells were maintained in Dulbecco modified eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 3.5 mg of glucose per ml and 10% fetal calf serum (GIBCO). Cells were split 1:6 into 24-well plates 3 days before the test. Cell density at the time of the test was 10^5 cells per cm^2. Test samples were dialyzed against phosphate buffer (0.02 M, pH 7.0) and passed through a 0.2-μm Acrodisc, and 100 μl was added to each well. Phosphate buffer controls were run concurrently with all tests. Cells were incubated in 5% CO_2 at 36°C and observed at intervals up to 12 h for disruption of the cell monolayer. Cytotoxicity was expressed as the reciprocal of the dilution that resulted in complete loss of activity.

Mouse-lethal toxin activity was determined by the injection of 0.1 ml of material in phosphate buffer (0.02 M, pH

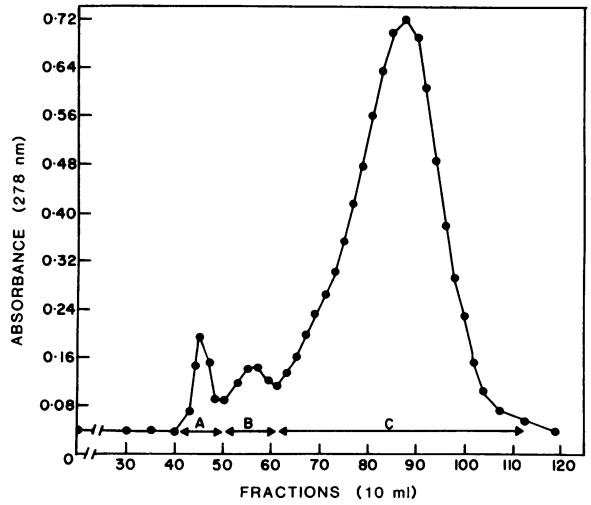

FIG. 2. Separation obtained by chromatography of the QAE(0.1) material on a column (4 by 100 cm) of Sephadex G-75 at a flow rate of 25 ml/h. Regions labeled A, B, and C represent fractions G75(A), G75(B), and G75(C), respectively, described in the text.

7.0) into the caudal vein of two ICR (Harlan-Sprague-Dawley, Indianapolis, Ind.) mice. Death within 30 min was considered to be a positive response.

Hemolytic activity was determined by mixing equal volumes of sample serially diluted in phosphate buffer (0.02 M, pH 6.8) containing 0.15 M NaCl and some trace metal ions with a 2% suspension (in 0.15 M NaCl) of washed rabbit erythrocytes. The assay was incubated at 37°C for 60 min, and the activity was expressed as the reciprocal of the titer giving complete clearing of the erythrocytes.

Protein estimation. The amount of protein present in the partially purified samples was estimated by optical density readings at 278 nm, assuming an extinction coefficient of 10.0.

Antiserum production. Antigens in phosphate buffer (0.02 M, pH 7.0) containing 0.15 M NaCl were emulsified with an equal volume of Freund complete adjuvant (Difco) and injected subcutaneously into the shoulders of female New Zealand White rabbits weighing 2 to 3 kg. Injections of increasing concentrations of antigen were generally made on days 0, 3, 8, 21, and 24. Test bleedings were performed and assayed for reaction with the antigen by double gel diffusion assays. Animals were boosted after a 3-week rest period with antigen emulsified in Freund incomplete adjuvant (Difco).

Serological detection of antigens. The antigens present in samples or dilutions of samples were detected by immunoprecipitation reactions in double gel diffusion assays by the method of Robbins et al. (20), except that 1.2% purified agar (Difco) in barbital buffer (0.04 M, pH 7.4) containing 0.15 M NaCl and merthiolate (1:10,000 dilution) was used.

Neutralization studies. Preliminary studies indicated that a component of normal rabbit serum interfered with the manifestations of the VP and Vero cell response. This component was not precipitated by 35% $(NH_4)_2SO_4$. Therefore, the immunoglobulin G-containing fraction of each antiserum was recovered by precipitation with 35% $(NH_4)_2SO_4$. Precipitates were redissolved in one-half the original volume and dialyzed overnight against phosphate buffer (0.02 M, pH 7.0). For neutralization studies, the toxic material in the same phosphate buffer was mixed with an equal volume of prepared antibody and incubated overnight at 4°C. The resulting immunoprecipitates were removed by centrifugation, and the supernatant fluids were assayed. The precipitated fraction from normal rabbit serum was run as a control in all experiments.

SDS-PAGE. Degree of purity of samples and estimation of molecular weights of proteins were determined by discontinuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). Electrophoresis was performed in a Protean Double Slab Electrophoresis Cell (Bio-Rad Laboratories, Richmond, Calif.), with a 10.5% running gel (1.5 by 140 by about 125 mm) prepared by the method of Laemmli (14). Low-range molecular weight standards were obtained from Pharmacia. Gels were stained overnight in a 0.12% solution of Coomassie blue R-250 (Bio-Rad) in ethanol-acetic acid-water (250:80:670) and destained overnight in the same ethanol-acetic acid-water solution.

Isoelectric focusing. The isoelectric point (pI) of each antigen was estimated by focusing 50 μg of each fraction in tubes (5 by 100 mm) of 11.25% polyacrylamide gel containing 6.25% narrow range (pH 4.0 to 6.5) Ampholine (LKB Instruments Inc., Rockville, Md.). After focusing at constant voltage (1.0 kV), the gels were frozen and cut into 2-mm segments. Each segment was eluted overnight with 0.5 ml of boiled deionized water, assayed for pH, and reacted with specific antisera to locate the antigens. A control gel, containing the antigens, was run concurrently and stained with 0.12% Coomassie blue to ensure that the samples had focused.

RESULTS

Initial isolation steps. Preliminary studies indicated that the RIL-active material was anionic in nature at pH 6.0 and above, but attempts to remove the factor from the large volume of culture supernatant by using Amberlite CG-400 as the adsorbent in batch chromatography were unsuccessful. The RIL and VP activity remained in the supernatant fluid; no activity could be detected in material eluted from the resin with up to 2.0 M NaCl. However, this procedure did remove many anionic species as evidenced by the large amount of UV-absorbing material that was adsorbed by the resin (Table 1). Table 1 contains an estimation of recovery of material and the specific activity present (as measured by the VP assay) at each step in the isolation procedure.

The toxic factor was adsorbed by batch chromatography with QAE-Sephadex. Differential elution of the ion exchanger with 0.05, 0.10, and 0.20 M NaCl resulted in the isolation of the RIL-active material in the 0.10 M NaCl elution volume (Table 2). This fraction [designated QAE(0.1)] also demonstrated VP and cytotoxic activity. However, the QAE(0.05) and QAE(0.2) eluents also showed considerable VP and cytotoxic activities, but no RIL activity. Most of the hemolytic activity was contained in the QAE(0.05) fraction, although some of this activity was also present in the QAE(0.1) and QAE(0.2) fractions. Most of the protease activity was present in the QAE(0.2) fraction. Because only the QAE(0.1) material showed RIL activity, this material was subjected to further separation procedures.

Chromatography on Sephadex G-75. The QAE(0.1) material was concentrated and chromatographed on Sephadex G-75; the resulting separation is indicated in Fig. 2. Major peaks were concentrated and tested for biological activity (Table 2); RIL, VP, cytotoxic, and hemolytic activities could be demonstrated only in peak B. This material [designated G75(B)] was analyzed by SDS-PAGE and found to contain three major protein bands and some minor bands (Fig. 3).

TABLE 1. Approximate recovery of each antigen[a] believed to be associated with toxic activities of *B. cereus* culture filtrates

Chromatographic fraction	Total protein (mg)[b]	Sp act of VP assay[c]	Antigens (mg)[d]	
			580	575
Crude material	21,000	N(36), B(73)	6.0 (100)	24 (100)
CG400 (void)	5,088	N(60), B(241)	6.0 (100)	24 (100)
QAE(0.1)	294	N(261), B(1044)	4.8 (80)	19 (79)
G75(B)	9.5	N(800), B(3200)	1.9 (32)	3.8 (16)
HA(0.06)	1.9		0.96 (16)	
HA(0.1)	2.6	N(800), B(1600)	0.24 (4)	1.0 (4)
HA(0.2)	0.61			0.5 (2)
HA(0.6 + 0.2)		N(1600), B(6400)		

[a] Estimated by serological detection.

[b] Total protein obtained from 12 liters of culture filtrate.

[c] N, necrosis; B, bluing. Values within parentheses indicate the specific activity of the vascular permeability test. This was determined by dividing the reciprocal of the dilution at which the response was less than 1 mm in diameter by the total protein contained in the test material.

[d] Estimation of total antigen contained in each fraction when 12 liters of crude material were processed. Values within parentheses denote the percent recovery of the antigen based upon the total amount in the crude material.

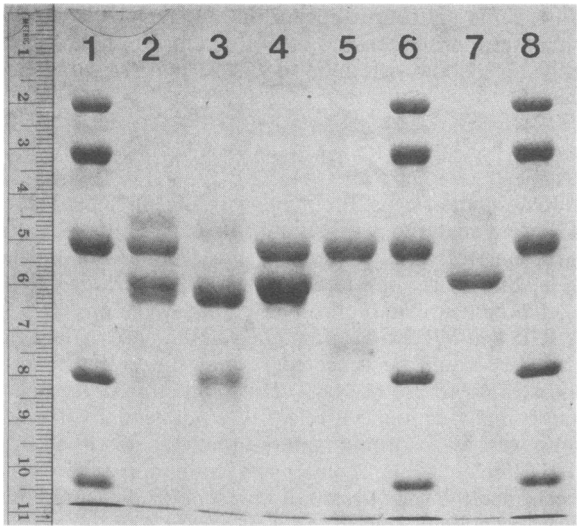

FIG. 3. Analysis of selected fractions by discontinuous SDS-PAGE. Channels 1, 6, and 8 contained molecular weight reference proteins; from the top of the gel these proteins were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). The remaining channels contained the following materials: 2, 130 μg of G75(B); 3, 50 μg of HA(0.06); 4, 100 μg of HA(0.1); 5, 50 μg of HA(0.2); 7, 50 μg of DEAE(B).

Antiserum prepared against the G75(B) material gave two precipitin lines with this fraction; the antigens that reacted with this serum were designated 575 and 577.

Chromatography on hydroxylapatite. The toxic factor was further isolated from the G75(B) material by chromatography on hydroxylapatite. Under conditions outlined above, about 30% of the UV-absorbing material did not adhere to the hydroxylapatite (Fig. 4). This material contained no detectable antigens when reacted with the antiserum produced against G75(B) and did not cause fluid accumulation in the RIL assay.

Stepwise elution of the hydroxylapatite with increasing phosphate concentrations (Fig. 4) recovered most of the rest of the protein applied to the hydroxylapatite column. These fractions were characterized with respect to antigens present and biological activity (Table 3). The protein contained in the fraction designated HA(0.06) was used to immunize a rabbit; the resulting serum allowed the detection of the third major protein present in the G75(B) material. This antigen was designated antigen 580. Analysis of the HA(0.06) fraction by SDS-PAGE showed two major bands and some minor bands (Fig. 3). Neither HA(0.04) nor HA(0.06) demonstrated any toxic activity.

The HA(0.1) fraction contained two of the three major SDS-PAGE bands present in the G75(B) material (Fig. 3). Serological analysis of this fraction revealed the presence of large amounts of antigens 575 and 577 and a smaller amount of antigen 580. When tested for biological activity, this fraction showed toxicity in all of the biological assays (Table 3). Threshold responses for the HA(0.1) material were 1.25 μg per injection for the necrotic response in the VP assay and 0.30 μg per ml in the Vero cell assay. The 50% effective dose in the RIL assay was about 300 μg of protein per loop. This fraction was also lethal to mice at a dosage of 1 mg per kg of body weight.

The HA(0.2) fraction contained one major band on SDS-PAGE and a large amount of the 575 antigen. A small amount of the 577 antigen could be detected serologically. This material did not cause fluid accumulation in RIL assays or give a response in the VP assay, but it did contain a small amount of cytotoxic activity in the Vero cell assay (Table 3).

Chromatography on DEAE-Sephacel. A variety of chromatographic procedures were tried in an effort to separate the 577 antigen from the 575 and 580 antigens. Separation was accomplished only when the G75(B) material was chromatographed on DEAE-Sephacel (Fig. 5). The 575 and 580 antigens eluted together in the first region [designated DEAE(A)]. The following region [designated DEAE(B)] contained the 577 antigen. A third region [designated DEAE(C)] contained no detectable antigens, with the exception of a small amount of the 577 antigen. However, when these fractions were tested for biological activity in the RIL, VP, and Vero cell assays, no activity could be detected. Combining the DEAE fractions did not restore activity.

Recombination of hydroxylapatite fractions. In an effort to examine combinations of the detectable proteins present in the toxic HA(0.1) material, the nontoxic, 580-containing HA(0.06) material was combined in equal proportions with the nontoxic, 575-containing HA(0.2) material. The resulting material [designated HA(0.06 + 0.2)] showed toxicity in all of the biological assays (Table 3). Threshold responses for the HA(0.06 + 0.2) material were 0.65 μg per injection for the necrotic response in the VP assay and 1.25 μg per ml in the Vero cell assay. The 50% effective dose in the RIL assay was 500 μg of protein per loop. This material was also lethal to mice after intravenous injection. The response of the HA(0.06), HA(0.2), and HA(0.06 + 0.2) fractions in the VP assay is shown in Fig. 6c through e. Complete activity could be recovered by injection of the HA(0.06) material followed immediately by the HA(0.2) material (Fig. 6f).

Neutralization studies. Hydroxylapatite fractions HA(0.06) and HA(0.2) were used to prepare antisera to antigens 580 and 575, respectively. Antiserum to the 577 antigen was prepared by using the DEAE(B) material. Antibody fractions were prepared as described above. The reconstructed

TABLE 2. Biological activities associated with fractions obtained from chromatography on QAE-Sephadex and Sephadex G-75

Fraction	RIL[a]	VP[b]	Vero cell[c]	Hemolysin[d]
QAE(0.05)	−(0/7)	N(8), B(32)	8	256
QAE(0.1)	+(5/7)	N(128), B(512)	128	128
QAE(0.2)	−(0/7)	N(128), B(512)	512	64
G75(A)	−(0/5)	−	−	2
G75(B)	+(4/5)	N(8), B(32)	16	32
G75(C)	−(0/5)	−	−	0

[a] Values within parentheses designate the number of positive responses over the number of animals considered valid in that test. QAE fractions were concentrated about 40 times; G75 fractions were tested at approximately 600 μg of protein per loop.

[b] N, Necrosis; B, bluing. Values within parentheses represent the reciprocal of the dilution at which the response was less than 1 mm in diameter. QAE fractions were concentrated about 40 times; G75 fractions were tested at approximately 10 μg of protein per injection. A negative response denotes no change from the buffer control run concomitantly.

[c] Expressed as the reciprocal of the dilution at which complete disruption of the cell monolayer is no longer observed. A negative test denotes no change from the buffer controls run concomitantly. QAE fractions were concentrated about 40 times; G75 fractions were tested at 20 μg of protein per ml.

[d] Expressed as the reciprocal of the dilution giving complete clearing of erythrocytes in 60 min. QAE fractions were concentrated about 40 times; G75 fractions were tested at 20 μg of protein per ml.

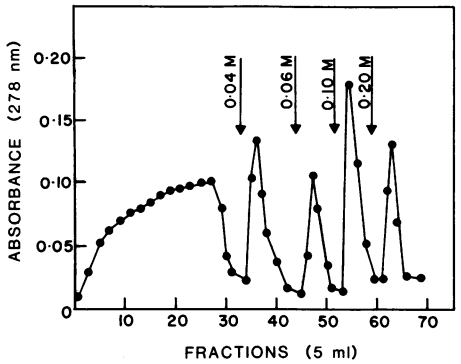

FIG. 4. Separation of G75(B) material by chromatography on a column (1.1 by 8.0 cm) by hydroxylapatite. Material was eluted stepwise with increasing concentrations of phosphate buffer (pH 7.0). Values and arrows indicate the changes in buffer concentration.

HA(0.06 + 0.2) material was reacted with each antibody preparation and tested in the VP and Vero cell assays. The results of these neutralizations are compiled in Table 4.

Antibodies prepared against the 575 antigen were capable of neutralizing both the necrotic and bluing responses of 2.5 μg of toxic material in the VP assay. This antibody preparation also neutralized the effect of up to 5.0 μg in the Vero cell assay.

Antibodies prepared against the 580 antigen were capable of neutralizing the necrotic response, but not all of the bluing response of 2.5 μg of toxic material in the VP assay. This antibody preparation was not capable of neutralizing the effect of 2.5 μg in the Vero cell assay.

Antibodies prepared against the 577 antigen were not capable of neutralizing the necrotic or bluing response of 2.5 μg of toxic material in the VP assay. This antibody preparation was not capable of neutralizing the effect of 2.5 μg in the Vero cell assay.

Determination of molecular weight and pI of antigens. Molecular weights were estimated by SDS-PAGE by using the HA(0.2) fraction for the 575 antigen, the DEAE(B) fraction for the 577 antigen, and the HA(0.06) fraction for the 580 antigen. The migration of the major bands in these fractions was compared with migration of molecular weight standards (Fig. 3). By this method we have estimated the molecular weight of the 575 antigen to be around 43,000, the molecular weight of the 577 antigen to be around 39,500, and the molecular weight of the 580 antigen to be around 38,000.

Isoelectric points of these antigens were determined by isoelectric focusing in polyacrylamide gels. The pI of antigens 575 and 577 is around 5.3. The pI of antigen 580 could not be determined by this method because of the serological instability of the protein in this system.

Evaluation of monkey feeding trials as an assay for the diarrheal toxin. During the course of this study, we have had many occasions to compare the biological activities associated with various fractions (Tables 2 and 3). Samples that were positive in the RIL assay were tested for the ability to induce diarrhea in Rhesus monkeys. Inconsistencies in results obtained with these tests led us to reexamine the methods originally described for monkey feeding (13). This procedure consisted of intragastric administration of 10 ml of neutralizing buffer (10% sodium bicarbonate, 4% peptone, and 1 mg of carmine red per ml) followed by 40 ml of filtered BHI culture supernatant or ammonium sulfate-precipitated material that had been redissolved in phosphate buffer (0.1 M). Table 5 is a compilation of the various monkey feeding trials that we recently performed.

Although neither BHI nor the neutralizing buffer alone induced diarrhea, the combination of these two substances did induce diarrhea. We were able to trace the diarrhea-producing components to the combination of disodium phosphate in the BHI and sodium bicarbonate in the neutralizing buffer (Table 5). The mode of action of this seemingly synergistic effect is not immediately apparent.

Several experiments were performed without the use of a neutralizing buffer to determine whether RIL-positive material could induce diarrhea in monkeys. Culture supernatant was concentrated 18 times by dialysis against polyethylene glycol. Two milliliters of this material produced a positive RIL response in seven of seven rabbits. Ten milliliters of this material (equivalent of 180 ml of original culture supernatant) was fed to each of four monkeys; no monkey developed diarrhea.

In a separate experiment, the crude material was processed through the CG-400 and QAE steps of the purification procedure and concentrated by dialysis against polyethylene glycol. Each of four monkeys was fed the QAE(0.1) equivalent of 3 liters of original culture supernatant. Again, no monkey developed diarrhea.

DISCUSSION

This study describes a series of chromatographic procedures whereby an extracellular toxic factor produced by *B.*

TABLE 3. Characterization[a] of the fractions obtained by chromatography of the G75(B) material on hydroxylapatite

Fraction	Major antigens	Minor antigens	RIL[b]	VP[c]	Vero cell[d]	Hemolysin[e]	ML[f]
HA(0.04)	580		−(0/5)	NT[g]	–	NT	NT
HA(0.06)	580	577	−(0/5)	–	–	16	0/2
HA(0.1)	575, 577	580	+(7/12)	N(8), B(16)	64	64	2/2
HA(0.2)	575	577	−(0/5)	–	2	4	0/2
HA(0.06 + 0.2)[h]	575, 580	577	+(9/12)	N(16), B(64)	16	16	2/2

[a] See footnotes to Table 2 for explanation of assays.
[b] Fractions HA(0.04) and HA(0.1) were tested at 300 μg of protein per loop. Fractions HA(0.06), HA(0.2), and HA(0.06 + 0.2) were tested at 500 μg of protein per loop.
[c] Each fraction was tested at 10 μg of protein per injection.
[d] Each fraction was tested at 20 μg of protein per ml.
[e] Each sample was tested at 20 μg of protein per ml.
[f] Mouse-lethal test. Expressed as the number of mice that died over the number injected intravenously. Each fraction was tested at 1 mg per kg of body weight.
[g] NT, Not tested.
[h] Combined fraction contained equal proportions of HA(0.06) and HA(0.2).

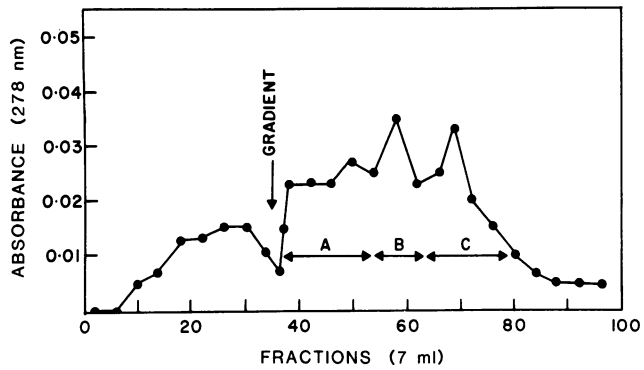

FIG. 5. Separation of G75(B) material by chromatography on a column (0.9 by 11.0 cm) of DEAE-Sephacel. Material was eluted by a gradient ranging from 0 to 0.175 M NaCl in the starting buffer. Regions labeled A, B, and C represent fractions DEAE(A), DEAE(B), and DEAE(C), respectively, described in the text.

cereus B-4ac and believed to be the agent responsible for diarrhea can be isolated. At least two dissimilar components seem to comprise this toxic factor. The isolation of this toxic factor was followed by a variety of biological assays.

By the various chromatographic steps described, we were able to isolate a fraction, designated HA(0.1), that demonstrated toxicity in the RIL, VP, Vero cell, and mouse-lethal assays (Table 3). Two other fractions, designated HA(0.06) and HA(0.2), did not show biological activity when tested individually, but all biological activities were restored by combining these two fractions (Table 3, Fig. 6). This reconstructed fraction was designated HA(0.06 + 0.2).

Antigen 580 was present as a major antigen in the nontoxic HA(0.06) fraction and as a minor antigen in the toxic HA(0.1) fraction. Antigen 575 was present as a major antigen in both the nontoxic HA(0.2) fraction and the toxic HA(0.1) fraction. Antigen 577 was present as a major antigen in the toxic HA(0.1) fraction and as a minor antigen in both the nontoxic HA(0.06) and HA(0.2) fractions. At one point we suspected that the 577 antigen was the toxic factor. However, some activity should have been detected in the HA(0.06) fraction if the 577 antigen were responsible for toxicity either alone or in combination with the 580 antigen. Similarly, more toxicity than the slight Vero cell activity (Table 3) should have been detected in the HA(0.2) fraction if the 577 antigen were responsible for toxicity either alone or in combination with the 575 antigen. By combining the nontoxic HA(0.06) and HA(0.2) fractions, we reconstructed a toxic fraction that contained the 575 and 580 antigens. Both of these antigens were also present, although in different proportions, in the toxic HA(0.1) material (Table 3).

The HA(0.06) material contained two major bands in SDS-PAGE (Fig. 3), but only one precipitin line could be detected when the antiserum prepared against this material was reacted with the antigenic material. We were able to separate a very small amount of the two proteins contained in this fraction by high-pressure liquid chromatography. The first peak contained the 580 antigen; the second peak contained material that did not react with the antiserum prepared against the HA(0.06) fraction. Cytotoxicity could only be detected when the HA(0.2) material was reacted with the peak containing the 580 antigen (data not shown). Because the particular column used with the high-pressure liquid chromatography separated proteins by molecular weight, we were able to correlate the early eluting 580 antigen with the larger-molecular-weight band on SDS-PAGE.

Because of these results, we believe that at least two components are needed for the manifestation of toxicity, and these components probably are the antigens that we have designated 575 and 580. Antigen 577 was present as a major antigen in the toxic HA(0.1) material and as a minor antigen in the toxic HA(0.06 + 0.2) material. It is possible that this antigen also contributes to toxicity. However, antibodies raised against the 577-containing DEAE(B) material (Fig. 5) did not neutralize the VP or Vero cell activities of the toxic HA(0.06 + 0.2) fraction (Table 4). Antibodies raised against the HA(0.06) and HA(0.2) fractions were capable of neutralizing the responses seen in the VP assay; antibodies raised against the HA(0.2) fraction were also capable of neutralizing the Vero cell activity of this material (Table 4). Specific antibodies to the 575 antigen were prepared by chromatographing the DEAE(A) fraction on hydroxylapatite. Antibodies to this HA(0.2) fraction were also capable of neutralizing the VP and Vero cell activities of the HA(0.06 + 0.2) material (data not shown).

Chromatography of the toxic G75(B) material on DEAE-Sephacel resulted in separation of the 577 antigen from the 575 and 580 antigens, but did not yield a toxic fraction. Fraction DEAE(A) contained antigens 575 and 580. By combining DEAE(A) with HA(0.06), we have been able to restore some, but certainly not all, of the Vero cell activity (data not shown). We cannot readily explain why chromatography on DEAE-Sephacel should irreversibly alter the toxic nature of the molecules. However, although controlled stability studies have yet to be performed, we have noticed considerable instability of the 580 antigen when it is isolated in the HA(0.06) fraction. This instability is manifested in the need to replace the refrigerated serological reagents almost on a daily basis. In addition, this antigen seems to be serologically unstable in our isoelectric focusing system.

The isoelectric point of antigens 575 and 577 was found to be around 5.3. We suspect that the pI of antigen 580 will be quite similar to that of 575 because both of these antigens

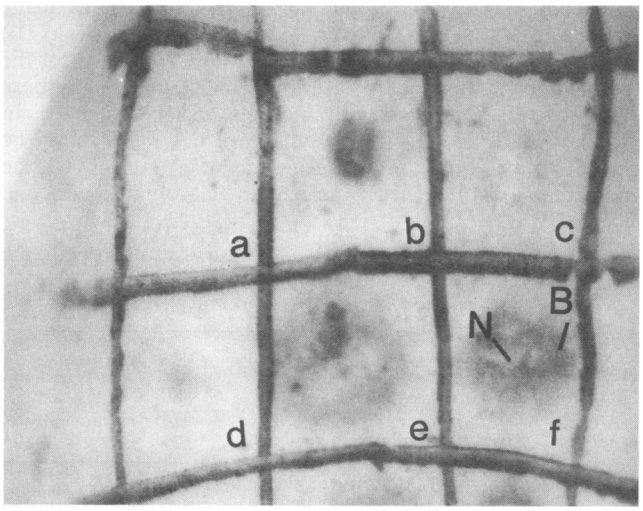

FIG. 6. Vascular permeability response of rabbit skin to hydroxylapatite fractions. Response is characterized as a necrotic zone (N) surrounded by a zone of bluing (B). Lettered areas received the following materials: a, 50 μl of phosphate buffer; b, 50 μl of crude material (positive control); c, 5.0 μg of fraction HA(0.06); d, 5.0 μg of fraction HA(0.2); e, 5.0 μg of the reconstructed fraction HA(0.06 + 0.2); f, 2.5 μg of fraction HA(0.06) followed immediately by 2.5 μg of HA(0.2).

TABLE 4. Abilities of antibodies prepared against various fractions to neutralize the VP and Vero cell activities[a] of the reconstructed toxic HA(0.06 + 0.2) fraction

Antibody prepn	VP[b]		Vero cell[c]	
	2.5 μg	0.65 μg	5.0 μg	2.5 μg
Normal rabbit serum	N(4), B(16)	B(4)	4	2
Anti-HA(0.06)	B(2)	–[d]	4	2
Anti-HA(0.2)	–	–	–	–
Anti-DEAE(B)	N(4), B(16)	B(2)	4	2

[a] See footnotes to Table 2 for explanation of activities.
[b] The effect of each antibody preparation on the VP activity when 2.5 and 0.65 of μg protein per injection were tested.
[c] The effect of each antibody preparation on the cytotoxicity when 5.0 and 2.5 μg per ml were tested.
[d] Negative response denotes no reaction was observed to that preparation.

were contained in DEAE(A) (Fig. 5). Turnbull et al. (24) reported the isolation, by isoelectric focusing, of a fraction that was VP positive and had a pI of 4.9. The reference by Turnbull (23) to unpublished observations that this isoelectric focusing fraction contained three proteins discernible by SDS-PAGE suggests that these investigators might have been dealing with a fraction containing the 575, 577, and 580 antigens described in this study. Their fraction demonstrated residual hemolytic activity. The toxic HA(0.1) and HA(0.06 + 0.2) fractions also demonstrated residual hemolytic activity (Table 3).

The toxic factor causes fluid accumulation in the RIL assay and by definition (2) can be considered as an enterotoxin because it acts directly on the intestine. The RIL assay is used as a valid assay for diarrheal toxins produced by *V. cholerae*, *E. coli*, and *C. perfringens*. All chromatographic fractions were tested in the RIL assay (Tables 2 and 3). The 50% effective dose of the toxic HA(0.1) fraction was about 300 μg of protein per loop. The 50% effective dose of the reconstructed toxic HA(0.06 + 0.2) fraction was about 500 μg of protein per loop. Perhaps this discordance reflects some of the instability in the toxin components when they are isolated.

All chromatographic fractions were tested also for the ability to increase permeability in the VP assay (Tables 2 and 3). The major problem with the VP assay is interpretation of the zones of bluing and necrosis. Generally, a necrotic response was surrounded by a zone of bluing (Fig. 6); occasionally, the bluing response was discernible as an outer zone of light blue surrounding an inner zone of dark blue. The latter situation was sometimes present with or without the central necrotic zone. Similar patterns have been noted by other investigators (12, 24). We have observed that dilution of necrotic material results in a dense zone of bluing. The observation that antibody prepared against the HA(0.2) fraction can neutralize both the necrotic and bluing response (Table 4) seems to indicate that necrosis is a potent manifestation of the bluing response, as has been suggested by Turnbull et al. (24). The endpoint of the necrotic reaction was about 1.25 and 0.65 μg of protein per injection for the HA(0.1) and HA(0.06 + 0.2) fractions, respectively. A very high correlation exists between the ability to cause fluid accumulation in the RIL assay and the ability to cause necrosis in the VP assay (12, 24; Tables 2 and 3 in this study). However, some fractions that caused an increase in VP activity did not cause fluid accumulation in the RIL assay (Table 2). Some of the 575 and 580 antigens could be detected in the QAE(0.05) and QAE(0.2) fractions; however, we cannot discount the possibility that other factors such as hemolysins and proteases might have contributed to the VP test in these fractions. Surely, it would have been misleading if the VP test alone had been used to analyze these fractions.

We have described the use of a cell culture assay for the detection of the toxic factor and have demonstrated a high correlation between cytotoxicity in the Vero cell assay and the positive RIL assay (Tables 2 and 3). The endpoint of the cytotoxicity was 0.30 and 1.25 μg of protein per ml of the HA(0.1) and HA(0.06 + 0.2) fractions, respectively. However, we are concerned about the inability to neutralize the Vero cell activity with antibodies prepared against the 580-containing HA(0.06) fraction (Table 4). This antibody preparation was capable of neutralizing the necrotic response of 2.5 μg and the bluing response of 0.65 μg of HA(0.06 + 0.2) in the VP assay. It is possible that a very small amount of the 580 antigen is needed to cause cytotoxicity in the Vero cell assay; this small quantity might have escaped neutralization by the antibody. We have not examined the HA(0.06) and HA(0.2) fractions with respect to the proportions of each that are needed to elicit a response in the various assays.

The toxic HA(0.1) and HA(0.06 + 0.2) fractions were also lethal to mice after intravenous injection. All fractions were tested at a dosage of 1 mg/kg of body weight; thus a threshold dosage was not determined. The relationship of our antigens to the multicomponent mouse-lethal toxin of Molnar (16) or to the 55,000- to 60,000-dalton toxin of Ezepchuk and Fleur (9) is not apparent because of the lack of characterization of the toxins in these previous studies. Spira and Goepfert (22) noted that their VP-positive chromatographic fraction was also lethal to mice.

Of the biological tests examined, the monkey feeding test proved to be the least dependable. We found that the buffering systems used in the original monkey feeding studies (13) caused diarrhea in the absence of bacterial metabolites (Table 5). We were unable to elicit diarrhea with large amounts of crude or partially purified material in the absence of neutralizing buffer. However, we cannot discount the possibility that an insufficient amount of material was actual-

TABLE 5. Ability of various compounds to induce diarrhea in rhesus monkeys

Treatment	Monkeys with diarrhea/monkeys fed material
10 ml of neutralizing buffer[a] followed by 40 ml of sterile BHI	8/8
10 ml of sterile water followed by 40 ml of sterile BHI	0/8
10 ml of neutralizing buffer followed by 40 ml of sterile water	0/8
10 ml of neutralizing buffer followed by 40 ml of BHI dialyzed against distilled water	2/8
10 ml of neutralizing buffer followed by 40 ml of 0.017 M disodium phosphate[b]	5/8
10 ml of water followed by 40 ml of 0.017 M disodium phosphate	0/4
10 ml of 10% sodium bicarbonate plus 1 mg of carmine red per ml followed by 40 ml of 0.017 M disodium phosphate	3/4
10 ml of 4% peptone plus 1 mg of carmine red per ml followed by 40 ml of 0.017 M disodium phosphate	0/4

[a] Neutralizing buffer contained 10% sodium bicarbonate, 4% peptone, and 1 mg of carmine red (gastrointestinal marker) per ml.
[b] BHI contains 0.017 M disodium phosphate.

ly reaching the target tissue in these experiments. Melling et al. (15) reported that the feeding of whole bacterial cultures of strain 4433 to monkeys produced diarrhea in 50% of the test animals; no mention of the use of a neutralizing buffer was made in their report. We suspect that humans develop diarrhea caused by *B. cereus* from toxin produced in the intestine after the ingestion of large numbers of bacterial cells. The relatively long incubation period (8 to 16 h) observed before the onset of diarrhea seems to substantiate this supposition.

We have given consideration to the seemingly large amount of toxin that is needed to elicit the various biological responses when compared with other bacterial toxins in similar systems. Perhaps the components of the *B. cereus* toxin were not present in the optimal proportions or the components were denatured during isolation, resulting in a product with low activity (or both). However, it is entirely possible that the *B. cereus* toxin just might not be a very potent toxin when compared with other bacterial toxins.

The precise relationship of the antigens described to toxicity has yet to be resolved. However, we believe that the restoration of RIL, VP, cytotoxic, and mouse-lethal activities by combining the nontoxic HA(0.06) and HA(0.2) fractions indicates that all of these activities probably are due to a single entity and that more than one component comprise this entity. Recently, Ohishi (19) reported that botulinum C_2 toxin induces fluid accumulation in mouse intestinal loops as well as increases vascular permeability and causes mouse lethality; this toxin is composed of two dissimilar, nonlinked protein components. Thus this structural relationship is not unique to the *B. cereus* toxin.

The realization that more than one component must be present seems to explain some of the difficulty that previous investigators encountered in attempting to isolate the *B. cereus* toxin. Many purification steps result in the separation of these proteins with consequent loss of biological activity. However, some of these difficulties might also be attributable to the apparent instability of the 580 antigen during isolation and storage.

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