Lethal Toxin of *Bacillus cereus*

I. Relationships and Nature of Toxin, Hemolysin, and Phospholipase

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*Bacillus cereus* phospholipase was characterized as a phospholipase C by the analysis of lecithin degradation products by thin-layer and paper chromatography. Methanol in the growth menstruum inhibited completely the synthesis of phospholipase C, whereas the synthesis of lethal toxin and hemolysin were only partially inhibited. Dialysis of preformed *B. cereus* products against ethyl alcohol and methanol did not inactivate hemolytic, phospholipase C, or lethal activity. The hemolytic and lethal activities of culture filtrates were completely abolished by trypsin, but phospholipase C activity was resistant to inactivation. Lethal and phospholipase C properties of culture filtrates were resistant to inactivation at 45°C, whereas the hemolytic activity was completely destroyed. Lethal, hemolytic, and phospholipase C activities appeared simultaneously in a complex growth menstrum, but the kinetics of synthesis were different in all cases. Resolution of *B. cereus* filtrates on columns of Sephadex showed that the phospholipase C, hemolysin, and lethal toxin are distinct proteins. Evidence is also presented which suggests a correlation between the synthesis of *B. cereus* toxin and the period of transition from vegetative growth to sporulation. The activity of each *B. cereus* product was cation-independent, as opposed to cation-dependency of the phospholipase C and lethal activities of *Clostridium perfringens* α-toxin. Immunological cross-reactivity between the *B. cereus* products and *C. perfringens* α-toxin was not apparent; indeed, they were shown to be antigenically distinct.

Bacterial phospholipases first assumed significance as potential virulence factors when it was established that the α-toxin of *Clostridium perfringens* is a true phospholipase (20). A comparison of the activities of *Bacillus cereus* culture filtrates with those of *C. perfringens* α-toxin led Chu (3) to speculate that in both cases a phospholipase enzyme is the lethal toxin. McGaughey and Chu (21) suggested further that the hemolytic, lethal, and lecithin-hydrolyzing capacity of *B. cereus* culture filtrates are all three the function of one molecular entity, namely, phospholipase C. On the contrary, Ottolenghi et al. (Bacteriol. Proc., p. 117, 1961) found that hemolytic and phospholipase activities of *B. cereus* culture filtrates exhibit a differential sensitivity to heat, suggesting nonidentity of the hemolysin and phospholipase. Since, in the latter study, toxin assays were not taken into consideration, the relationship among the three extracellular products of *B. cereus* was not brought into focus. Fractionation of *B. cereus* culture filtrates on columns of diethylaminoethyl (DEAE) cellulose by Fossom (7) and Slein and Logan (25, 26) suggested a distinction between the hemolysin and phospholipase C, but again no attempt was made to correlate the data with the quantity of lethal toxin present in the fractions. Molnar (22) reported the separation of lethal toxin of *B. cereus* into two subunits (factors I and II) on columns of calcium-phosphate gel, with neither factor alone exhibiting toxicity for mice. The combination of factors I and II formed a toxic mixture which was lethal for mice. Phospholipase C activity as determined by the lecitho-vitellin reaction (17) was found only in the sample which contained factor I.

The present investigation therefore was conducted to establish the relationship among the *B. cereus* hemolysin, lethal toxin, and phospholipase and demonstrates convincingly that the three extracellular activities are catalyzed by distinct proteins.

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MATERIALS AND METHODS

Organism and cultural conditions. B. cereus B-48, obtained from the stock culture collection of the Department of Microbiology, University of Cincinnati College of Medicine, was employed for these experiments and was assumed to be representative of the species. A preliminary survey of 10 strains of B. cereus showed that all of them produced lethal toxin and phospholipase(s) under the appropriate cultural conditions (2). A loopful of growth from a stock culture slant was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of medium, and was incubated on a reciprocating-shaker water bath at 84 cycles/min at 37 C for 24 hr. A subculture was continued for 16.5 hr, and the cells were harvested by centrifugation at room temperature. The cells were washed twice with sterile physiological saline and suspended in sterile 0.15% agar to a Klett reading of 330 in a Klett-Summerson colorimeter (640 to 700 m). As a standard inoculum volume 0.5 ml of the standard cell suspension was used per 50 ml of medium.

Determination of bacterial growth. Growth was determined turbidimetrically. Duplicate samples were taken at designated times, and turbidity was determined in a Klett-Summerson colorimeter (640 to 700 m). The samples were centrifuged and the supernatant fluids were used for toxin, hemolysin, and phospholipase assays.

Concentration of culture filtrates. Bacteria-free culture filtrates prepared by centrifugation and membrane filtration were added to dialysis sacs (Visking tubing), immersed in a slurry of Carbowax (polyethylene glycol 2-M, Union Carbide Co., South Charleston, W.Va.), and placed at 4 C overnight. After concentration, the sacs were opened by truncation, and the concentrated filtrate was brought to the desired volume by the addition of borate buffer (0.05 M, pH 7.0). Occasionally, sterile filtrates were precipitated with ammonium sulfate (60 and 100% saturation) and concentrated as described above.

Titration of B. cereus lethal toxin. The mouse lethality test was used for the titration of B. cereus toxin (22). Twenty-gram white mice were challenged intravenously via a caudal vein with serial twofold dilutions of toxin in gelatin-saline. A volume of 0.5 ml was adopted as the standard challenge dose. Four animals were challenged with each dilution, and LD50 values are reported as the reciprocal of the highest dilution of sample which killed 50% of the animals within 30 min.

Titration of B. cereus hemolysin. The hemolytic activity of B. cereus hemolysin was measured by the complete hemolysis of rabbit erythrocytes. Twofold serial dilutions of samples were made in sterile physiological saline. An equal volume of 0.5% (v/v) rabbit erythrocytes was added to each tube, and the mixture was incubated statically in a water bath (37 C) for 1 hr. Hemolytic titers are expressed as the reciprocal of the highest dilution of a sample which resulted in complete hemolysis in 1 hr at 37 C. The smallest amount of hemolysin necessary for complete hemolysis in 1 hr at 37 C is defined as 1.0 unit of hemolysin.

Determination of phospholipase activity. To measure the lecitho-vitellin reaction, samples of sterile culture filtrates, usually 0.5 ml, were added to 4.5 ml of egg-yolk lecithin (21) and incubated statically at 37 C. In the preparation of egg-yolk lecithin, borate buffer (0.05 M, pH 7.4) with CaCl2 (2.5 mm) was substituted for nutrient broth. At appropriate intervals, turbidity was determined in a Klett-Summerson photoelectric colorimeter (640 to 700 m).

To determine the acid-soluble phosphorus content, duplicate 0.5-ml samples of culture filtrates or fractions from columns were added to sterile tubes containing 4.5 ml of egg-yolk lecithin. After various times, the enzymatic reaction was stopped by the addition of an equal volume of cold 10% trichloroacetic acid. The aqueous phase was separated from protein and unreacted substrate by filtration through either Whatman no. 42 or Schleicher and Schuell no. 602 filter paper. The acid-soluble phosphorus content of the filtrate was determined by the method of King (15).

Chromatographic methods for detection of enzymatic degradation products of lecithin. Descending paper chromatography was used for the detection of phosphorylcholine. The methods of Hanes and Isherwood (8) and Dawson (6) were employed. Phosphorylcholine (Nutritional Biochemicals Corp., Cleveland, Ohio) was used as the control. Chromatograms were removed from the chamber after 10.5 hr, dried, and sprayed with the developing agent of Hanes and Isherwood (8). The chromatograms were dried under a hood and heated to 80 C for 10 to 15 min in an air oven for the development of color.

Thin-layer chromatography was used to detect diglycerides and triglycerides. Plates of Silica Gel G (20 by 20 cm) with a gel depth of 0.25 mm were kindly provided by J. M. Iacono, Cincinnati General Hospital, Cincinnati, Ohio. Samples were spotted with a 50-μl syringe, and the plates were air-dried. The solvent system was hexane-diethyl ether-acetic acid (80:20:1). The plates were placed in a solvent chamber and, after 45 min, were removed, air-dried, placed under a hood, and sprayed with a 0.05% solution of Rhodamine B. Fluorescence under ultraviolet light indicated the presence of lipids. The fluorescent spots were traced onto onion skin paper to provide permanent records.

Production of antisa. To produce anti-phospholipase, 1 ml of crude B. cereus phospholipase (Johnson and Bonventre, Bacteriol. Proc., p. 41, 1966) was added to an equal volume of Freund's complete adjuvant and was emulsified by treatment in a Vortex mixer for 15 min. Rabbits were injected subcutaneously in the region of the hip once a week for three consecutive weeks with 0.5 ml of the emulsion. After the last injection, the rabbits were rested for 14 days, then bled by cardiac puncture. The same procedure was employed for the production of anti-C. perfringens α-toxin (Sigma Chemical Co., St. Louis, Mo.). To produce anti-B. cereus toxin, 1 ml of crude toxin (which contained 5 to 10 mouse LD50 doses) was mixed with an equal volume of Freund's complete adjuvant.
The immunization schedule was the same as that described for the phospholipases.

**RESULTS**

**Preliminary nutritional studies.** The growth of *B. cereus* was supported by all of the complex, semisynthetic, and synthetic media tested. The complex media employed were Trypticase Soy Broth (BBL), Nutrient Broth (Difco), and beef infusion prepared from fresh beef. Vitamin Free Casamino Acids (Difco), acid-hydrolyzed Casamino Acids (Difco), and Hy-Casamino Acids (Sheffield Chemical Co., Norwich, N.Y.) at a final concentration of 1.25% (w/v) were employed as semisynthetic media. A synthetic medium composed of 18 amino acids (28) and a mineral-salts mixture was also tested.

Consistent production of significant amounts of toxin was observed only when *B. cereus* was cultured in fresh beef infusion. In the other growth media, toxin either was produced in small quantities or not at all. Toxin production was not enhanced by supplementation of growth media with glucose or with other carbohydrates, proteose-peptone, peptides, or B vitamins, either singly or in combination. Indeed, no exogenous metabolite with the capacity to stimulate lethal toxin production was found.

**Characterization of *B. cereus* phospholipase.** Present acceptable nomenclature defines a phospholipase C as an enzyme which liberates phosphorylcholine and a diglyceride from a lecithin substrate (13). The analysis of lecithin degradation products derived from the interaction of the *B. cereus* enzyme and a lecithin substrate therefore was used for the characterization of the bacterial phospholipase. The enzyme was a lyophilized culture filtrate devoid of lethal and hemolytic activities (Johnson and Bonventre, Bacteriol. Proc., p. 41, 1966) reconstituted to its original volume (5.0 ml) with borate buffer (0.05 M, pH 7.4). *C. perfringens* α-toxin (0.5 mg/ml; Sigma Chemical Co.) was used as a control phospholipase C. Each enzyme preparation (0.5 ml) was added to a complex lecithin (egg-yolk lecithin) substrate and incubated statically in a water bath at 37°C; 20 μlitrers of the reaction mixture were spotted on plates of Silica Gel G. The following materials were employed as controls: a mixture of tripalmitin, palmitic acid, methyl stearate, and cholestel stearate (each at a concentration of 4.0 μg/ml of CCl₄) and a diglyceride, 1,3-dipalmitin (2 mg/ml of CCl₄); a monoglyceride, mono-olein (2 mg/ml of CCl₄); and cholesterol (2 mg/ml of CCl₄). The egg-yolk lecithin substrate and both enzymes were also spotted and served as additional experimental controls.

Neither the *B. cereus* enzyme nor α-toxin contained contaminating lipid materials. Cholesterol and triglycerides but not diglyceride or free fatty acids were present in the complex lecithin substrate. The 1,3-dipalmitin control produced two contiguous spots which suggested either impurity of the diglyceride or isomerization of 1,3-dipalmitin to 1,2-dipalmitin. The second explanation was considered more likely than the first, since the polarity of 1,3-dipalmitin is less than that of 1,2-dipalmitin and the former would be expected to migrate farther from the origin (J. M. Iacono, personal communication). Of the two contiguous spots which resulted from the isomerization of dipalmitin, the one nearer the origin corresponded to the RF of a substance released from the complex lecithin substrate by the action of both the *B. cereus* enzyme and α-toxin. This evidence showed that one of the products which resulted from the interaction between the bacterial enzymes and the lecithin substrate was a diglyceride. The absence of free fatty acids and other products of lecithin degradation indicated that the *B. cereus* enzyme preparation did not contain either lipases or phospholipases A, B, and D. The enzymatic activity of culture filtrates of *B. cereus* was not influenced by hemolytic or lethal activities. A culture filtrate which demonstrated lethal and hemolytic properties in addition to phospholipase activity reacted in the same fashion with the lecithin substrate as did the nonlethal preparation. The results of an enzymatic interaction between a synthetic lecithin substrate (L-α-lecithin, dipalmitoyl; General Biochemicals, Chagrin Falls, Ohio) and α-toxin or *B. cereus* filtrates (lethal or nonlethal) were comparable to those obtained with the complex lecithin substrate.

To establish that the *B. cereus* enzyme was a phospholipase C, it was also necessary to demonstrate phosphorylcholine as an enzymatic degradation product. The reaction mixtures which were used for the thin-layer chromatography assay for diglyceride were filtered through Whatman no. 42 filter paper; 100 μlitrers of each filtrate was spotted on strips of Whatman no. 1 chromatography paper. Controls included 15 μlitrers of a 0.2% phosphorylcholine solution (Nutritional Biochemicals Corp.), 100 μlitrers of the lecithin substrate, 50 μlitrers of *B. cereus* enzyme, and 50 μlitrers of a 0.05% solution of α-toxin. Descending paper chromatography was performed by the method of Dawson (6). A substance with the same RF value as phosphorylcholine was evident in all filtrates tested. Phosphorylcholine was absent from substrate and enzyme controls.
The demonstration of diglycerides and phosphorylcholine as products of the enzymatic interaction between lecithin and the *B. cereus* enzyme show conclusively that the phospholipase elaborated during growth is a phospholipase C.

**Effect of alcohols on the synthesis and activities of *B. cereus* products.** It has been shown that the addition of low-molecular-weight alcohols to growth media inhibits the synthesis of phospholipase C by *B. cereus* (18, 19) and α-toxin by *C. perfringens* (14). This was used as a means of differentiating the extracellular products of *B. cereus*. Methanol concentrations of 2.0 to 4.0% inhibited the synthesis of *B. cereus* phospholipase C in both synthetic and complex media as determined by the lecitho-vitellin reaction. On the contrary, methanol did not inhibit appreciably the synthesis of lethal toxin. Since there may be inconsistencies between the activity of *B. cereus* phospholipase C and the amount of turbidity engendered in the lecitho-vitellin reaction (17), the effect of methanol on the synthesis of *B. cereus* products was also evaluated by using acid-soluble phosphorus released from a complex lecithin substrate as a measure of enzyme activity.

The synthesis of phospholipase C was inhibited completely by methanol (4%, v/v), but the synthesis of lethal toxin and hemolysin was only partially inhibited by the solvent (Table 1). The less-than-normal quantity of lethal toxin and hemolysin obtained may have been the result of two concurrent mechanisms: (i) an inhibition of toxin and hemolysin synthesis, and (ii) an alteration of the cytoplasmic membrane of the microorganism which would account for the delayed appearance of the two products in the growth menstrum. The specific and complete inhibition of synthesis of *B. cereus* phospholipase C by methanol, however, suggested that the enzyme was distinct from the lethal toxin and hemolysin.

The apparent inhibition of the synthesis of *B. cereus* products by methanol could conceivably have been a reflection of their inactivation upon release into the growth menstrum. Consequently, attempts were made to differentiate between the inhibition of synthesis and inactivation of preformed *B. cereus* products by the low-molecular-weight solvents; 10 ml of culture filtrate was added to each of three dialysis sacs and dialyzed at 4°C against borate buffer (0.05 M, pH 7.0), 30% methanol, and 30% ethyl alcohol, respectively. After 6 hr, the solvents were replaced with 2 volumes of borate buffer, and dialysis was continued for an additional 18 hr. Dialysis against methanol and ethyl alcohol did not inactivate any of the three preformed products of *B. cereus* (Table 2).

**Effect of trypsin, hydrogen-ion concentration, and heat on the activities of *B. cereus* products.** Slein and Logan (25) described a *B. cereus* enzyme which utilized both phosphatidyl choline (lecithin) and phosphatidyl ethanolamine as substrates and which was resistant to the action of trypsin. We employed exposure to this proteolytic enzyme as another means of differentiating the *B. cereus* products. It was observed that the lethal and hemolytic properties of culture filtrates were destroyed completely by trypsin (Fig. 1). Since the rates of inactivation of both activities were quite similar, no distinction could be made between lethal toxin and hemolysin. The activity of phospholipase C, however, was unaltered after 2 hr of exposure to trypsin.

**Table 1. Phospholipase C, lethal, and hemolytic activities of culture filtrates of *Bacillus cereus* grown at 37°C in a complex medium containing 4% methanol.**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Medium</th>
<th>Growth*</th>
<th>Toxin (LD₅₀/ml)</th>
<th>Hemolysin (HU/ml)</th>
<th>Phospholipase C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>Infusion</td>
<td>0.264</td>
<td>6.72</td>
<td>512</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.420</td>
<td>11.32</td>
<td>512</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.210</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Infusion + methanol</td>
<td>0.374</td>
<td>8.0</td>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

* Optical density units.
* Hemolytic unit (HU) is defined as the least amount of hemolysin necessary for the complete hemolysis of a 0.5% rabbit erythrocyte suspension in 1 hr at 37°C.
* Expressed as micrograms of acid-soluble phosphorus per milliliter of trichloroacetic acid filtrate.
* Fresh beef infusion.

**Table 2. Effect of dialysis against alcohols on the lethal, hemolytic, and phospholipase C activities of culture filtrates of *Bacillus cereus*.**

<table>
<thead>
<tr>
<th>Dialysis menstrum</th>
<th>Inactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lethal toxin</td>
<td>Hemo-lysin</td>
</tr>
<tr>
<td>Methanol (30%)</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl alcohol (30%)</td>
<td>0</td>
</tr>
<tr>
<td>Borate buffer (control)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Filtrates were dialyzed against alcohol for 6 hr at 4°C. Dialysis was continued for an additional 18 hr against borate buffer before assay.
The difference in stability of *B. cereus* products under alkaline and acid conditions added data to the accumulating evidence for their nonidentity (Table 3). There was no observable decrease in the hemolytic activity under acid conditions, whereas there was a 75% diminution in activity under alkaline conditions. The toxin titer was diminished equally under both acid and alkaline environments, but the activity of phospholipase C was not appreciably influenced under either circumstance.

Ottolenghi (24) showed that the hemolytic activity of *B. cereus* filtrates was inactivated at 56 C, whereas the phospholipase C activity was unaltered. Our results corroborated those of Ottolenghi in that both hemolysin and toxin were rapidly inactivated at 56 C and the activity of phospholipase C remained unaltered. At 45 C, however, a differential inactivation of the hemolytic and lethal activities of *B. cereus* culture filtrates was observed (Fig. 2).

**Temporal synthesis of *B. cereus* extracellular products.** It was considered valid to assume that an identity among the activities of *B. cereus* filtrates would necessitate a consistent relationship among the titers of the three at any given time during growth. This assumption was examined experimentally by taking samples of growing cultures at intervals and assaying the filtrates for hemolysin, lethal toxin, and phospholipase C activities. Extracellular products possessing lethal and hemolytic properties and phospholipase C activity were detected concurrently in the growth

### Table 3. Effect of pH on the activity of *Bacillus cereus* culture filtrates

<table>
<thead>
<tr>
<th>pH</th>
<th>Lethal toxin</th>
<th>Hemolysin</th>
<th>Phospholipase C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>70</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>11.1</td>
<td>57</td>
<td>75</td>
<td>3</td>
</tr>
<tr>
<td>7.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Filtrates were held at the pH indicated for 24 hr at 4 C and then readjusted to pH 7.3 before assay.*

menstruum, but disappeared at differential rates (Fig. 3). The titer of hemolysin was maximal after 3 hr of incubation and remained unaltered for 24 hr. Between 24 and 48 hr, however, the hemolysin was markedly diminished. The titer of lethal toxin was maximal at 6 hr and remained constant until the termination of the experiment (48 hr). On the other hand, phospholipase C activity was maximal after 4 hr of incubation, but declined rapidly; only 19% of maximal activity was detectable 8 hr after inoculation.

This evidence corroborates previous data (7, 22, 25, 26) which suggested the presence of several distinct factors in culture filtrates of *B. cereus*.

**Correlation between toxin synthesis and sporulation.** The growth of *B. cereus* in appropriate media is characterized by a decrease in the pH of the growth menstruum, followed by an increase in alkalinity (9). These changes in pH have been correlated with the transition from vegetative growth to metabolism characteristic of sporulation (10). During our experiments we noted that the production of toxin occurred primarily
during the transitional phase of growth. This observation was evaluated by use of Trypticase Soy Broth, Nutrient Broth, and fresh beef infusion. Neither Trypticase Soy Broth nor Nutrient Broth supported the production of toxin by \textit{B. cereus}.

In fresh beef infusion, a correlation between an increase in extracellular toxin and the transitional phase of growth, as determined by the \( pH \), was observed (Fig. 4). The \( pH \) of fresh beef infusion remained constant at its lowest value (6.75) for 3 hr, during which the toxin titer reached a maximum. On the contrary, neither of the other two complex media showed a comparable pattern. In Trypticase Soy Broth, the \( pH \) decreased to 6.0, but it began to rise immediately after reaching this lowest level. The initial \( pH \) of Nutrient Broth remained constant for the first 3 hr of incubation, after which it demonstrated a rapid increase toward the alkaline range. These data would suggest a correspondence between biochemical events which contribute to maintaining an acid environment during the transitional phase of growth and the synthesis of lethal toxin by \textit{B. cereus}.

\textbf{Physical separation of \textit{B. cereus} products.} Attempts to separate the components of \textit{B. cereus} culture filtrates on columns of DEAE cellulose were unsuccessful. Although the hemolysin was recovered quantitatively, lethal toxin and phospholipase \textit{C} were apparently reduced to inactive subunits, since neither could be recovered in individual fractions. Recombined fractions possessed both lethal and phospholipase \textit{C} activities.
Reduction of lethal toxin to inactive subunits is consistent with the observations of Molnar (22). These data therefore did not confirm the individuality of toxin and phospholipase C, since each recombined fraction which possessed the property of lethality also expressed phospholipase C activity.

Filtration through Sephadex was also employed in an attempt to separate the products of B. cereus. Sephadex G-200 was prepared as suggested by the manufacturer and packed in a column (2.0 by 105 cm) by gravity at 4 C. A B. cereus culture filtrate was concentrated approximately 10-fold in Carbowax at 4 C, and a 5.0-ml sample was added to the column and "washed-in" with 5.0 ml of elution buffer (0.05 M borate buffer, pH 7.0). Elution buffer was delivered to the column under the pressure of a constant hydrostatic head of 35 cm, which effected a flow rate of 30 ml/hr. Under these conditions, the B. cereus products were not separated completely. However, fractions which exhibited maximal hemolytic activity possessed neither lethal nor phospholipase C activities. Fractions which contained all three activities were combined and concentrated in Carbowax. A 6.0-ml sample was added to a column of Sephadex G-75 (2.25 by 34 cm) for additional resolution. Elution buffer was delivered under pressure from a 20-cm hydrostatic head, which induced a flow rate of 15 ml/hr at 4 C. The results (Fig. 5) indicate that the lethal toxin, hemolysin, and phospholipase C are distinct entities. Although there was an overlapping of activities in some of the fractions, the possession of distinct activities by different proteins is without question. If the three biological properties were the function of a single protein species, the distribution of the hemolysin, phospholipase, and lethal toxin in fractions should have been present in a constant ratio to each other. The independent nature of B. cereus hemolysin and phospholipase C is clearly indicated by the gel-filtration data. Although the separation of hemolysin and lethal toxin was not as clear-cut, the nonidentity of these was established by a growth experiment which took advantage of the fact that dialyzed fresh beef infusion does not support the production of significant quantities of toxin by B. cereus.

Cultures of B. cereus were established in

![Fig. 5. Separation of toxin, hemolysin, and phospholipase C by gel filtration (Sephadex G-75). (X) Phospholipase C; 1 unit of enzyme is defined as the amount of enzyme required to liberate 100 μg of acid-soluble phosphorus from an egg-yolk lecithin substrate in 45 min at 37 C. (▲) Toxin, expressed as LD₅₀/5-ml fraction. (●) Hemolysin, expressed as log₂ units of hemolysin/ml of eluate.](http://jb.asm.org/DownloadedFrom/)
dialyzed and nondialyzed fresh beef infusion, and both toxin and hemolysin titers of the filtrates were measured during the first 6 h of incubation at 37°C (Fig. 6). Growth of the microorganism was essentially the same in both media. Microscopic examination of a 6-hr culture of B. cereus grown in dialyzed medium revealed intracellular spores, free spores, and cellular debris. No spores or cell lysis was evident in the 6-hr control culture (nondialyzed medium). The morphological evidence suggests that the release of enzymes upon the lysis of vegetative cells during sporulation may have been responsible for the precipitous decline in the hemolytic titer observed in the filtrates of cultures grown in dialyzed medium. The fact that the titer of toxin in the dialyzed medium differed from that in the control (nondialyzed medium) by approximately 2.5-fold, whereas the titers of hemolysin were identical under both circumstances, speaks against an identity of lethal toxin with hemolysin.

Biological and immunological properties of B. cereus filtrates as compared with C. perfringens α-toxin. The α-toxin of C. perfringens is characterized as being lethal, hemolytic, and phospholipolytic, all three activities being expressed by one protein. On the other hand, although B. cereus culture filtrates demonstrate biological activities which are identical with those of α-toxin, each activity is catalyzed by a distinct protein. In view of the fact that B. cereus filtrates and C. perfringens α-toxin exhibit comparable biological activities, a comparison was made of their properties as they relate to cation dependency and immunological specificity.

To evaluate the influence of divalent cations on the activity of B. cereus phospholipase C, chelators were added to egg-yolk lecithin to bind all divalent cations. Lyophilized B. cereus phospholipase C was reconstituted to its original volume (5.0 ml) in borate buffer (0.05 M, pH 7.0). C. perfringens α-toxin (0.5 mg/ml) served as a control. The chelators employed were diethylenetriamine pentaacetic acid (DTPA; Geigy Pharmaceuticals, Ardsley, N.Y.), ethylene diaminetetraacetic acid (Matheson, Coleman and Bell, Cincinnati, Ohio), and disodium ethylenediazonitratetraacetate dihydrate (Sequestrene; Geigy Pharmaceuticals). Chelators were added to the lecithin substrate to the desired concentration, and the substrate-chelator mixtures were incubated at 37°C for 15 min prior to the addition of enzymes.

All of the chelating agents, at a final concentration of 100 μg/ml of substrate, inhibited completely the phospholipase C activity of α-toxin (Table 4). The sequestration of divalent cations did not significantly inhibit the activity of B. cereus phospholipase C. Increasing the concentration of one chelator (Sequestrene) to 500 μg/ml of substrate did not significantly depress the activity of B. cereus phospholipase C. The evidence therefore indicates that B. cereus phospholipase C does not require free divalent cations for activity. These observations are in agreement with those of Chu (3), but are contrary to Ottolenghi's (24) report that B. cereus phospholipase C is a zinc-requiring enzyme.

To investigate the influence of divalent cations on the B. cereus toxin and hemolysin, DTPA was added to dilutions of a culture filtrate. α-Toxin (0.5 mg/ml), which served as a control, was treated similarly. All samples were incubated at 37°C for 15 min prior to toxin and hemolysin titrations. The lethal activity of α-toxin was inhibited 65% by DTPA at a concentration of 100 μg/ml (Table 4), but DTPA did not influence the lethal activity of the B. cereus filtrate. The hemolytic activity of C. perfringens α-toxin and B. cereus hemolysin both remained unaltered in the presence of DTPA. The failure of DTPA to influence the hemolytic activity of C. perfringens α-toxin may reflect either the presence on the protein molecule of multiple catalytic sites which vary in their cation requirement for activity, or the contamination of the commercial C. perfringens α-toxin preparation with β-toxin. The former hypothesis awaits substantiating evidence, and the latter possibility seems unlikely since the oxygen lability of β-toxin would probably have rendered this hemolysin inactive under our experimental conditions.

It is clear that in vitro requirements do not necessarily reflect the in vivo situation. Although
TABLE 4. Effect of chelating agents on the activities of Bacillus cereus products and Clostridium perfringens $\alpha$-toxin

<table>
<thead>
<tr>
<th>Activity</th>
<th>Chelator</th>
<th>Conc</th>
<th>Assay*</th>
<th>Inhibition</th>
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<tr>
<td></td>
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<td>$\mu g/ml$</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>DTPA$^a$</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sequestrene$^e$</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B. cereus$^d$</td>
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<td>4.6</td>
<td>0</td>
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<td>DTPA</td>
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<td></td>
<td>DTPA</td>
<td>100</td>
<td>64</td>
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* Phospholipase C is expressed as enzyme units per milliliter, with 1 unit being that amount of enzyme necessary to release 100 $\mu g$ of acid-soluble phosphorus from egg-yolk lecithin in 45 min at 37 C. Lethal toxin is expressed in terms of the LD$_{50}$ per milliliter. Hemolysin is given as hemolysin units per milliliter.

$^a$ Diethylenetriamine pentaacetic acid.

$^e$ Disodium ethylenediaminetetraacetic acid dihydrate.

$^d$ B. cereus phospholipase C was a lyophilized culture filtrate possessing neither hemolytic nor lethal activity.

The in vitro experiments failed to implicate a requirement for divalent cations for the activity of B. cereus toxin, the experiments were extended to determine whether the toxin required cations for activity in vivo. Seventy-five mice were given 1.0 mg (0.5 ml) of DTPA intraperitoneally every hour for 3 hr. A comparable regimen of physiological saline was administered to 20 control mice. One hour after the last injection of either the chelator or saline, four mice were challenged either by the intravenous or intraperitoneal route with different concentrations of $\alpha$-toxin or B. cereus toxin. Animals prepared with DTPA showed a 75% protection when challenged intravenously with 5.3 LD$_{50}$ of $\alpha$-toxin; animals challenged by the same route with 1.4 LD$_{50}$ of B. cereus toxin suffered 100% mortality (Table 5). The chelating agent completely protected mice challenged intraperitoneally with 3.5 LD$_{50}$ of $\alpha$-toxin, but did not protect against a challenge with 2 LD$_{50}$ of B. cereus toxin. The protection of animals from the lethal effects of $\alpha$-toxin by the administration of a chelating agent has been documented by Moskowitz (23). On the contrary, our data indicate that the in vivo activity of B. cereus toxin does not require cations for activity.

Immunological specificity of B. cereus products and C. perfringens $\alpha$-toxin. In view of the biochemical similarity of the extracellular products of B. cereus and the $\alpha$-toxin of C. perfringens, and the taxonomic relationship of the two species, it was considered of interest to examine the immunospecificity of their bacterial products.

Antisera were prepared against $\alpha$-toxin and B. cereus toxin, phospholipase, and hemolysin. Cross-reactivity of antigens was determined by a comparison of the specificity of homologous and heterologous antigen-antibody systems; 0.5 ml of undiluted homologous or heterologous antiserum was added to $\alpha$-toxin (40.8 LD$_{50}$), B. cereus toxin (24 LD$_{50}$), and a partially purified preparation of B. cereus phospholipase C, to give a final volume of 6.5 ml. The antigen-antibody mixtures were then incubated at 37 C for 1 hr. Lethal activity was determined by the mouse lethality test, and the lecitho-vitellin reaction was used as a qualitative index of phospholipase C activity. Undiluted normal rabbit serum served as a control to detect nonspecific inactivation.

Immunological specificity rather than cross-reactivity was demonstrated. Antisera against partially purified phospholipase C and toxin of B.
cereus did not inactivate the lethal or phospholipase C activities of C. perfringens α-toxin. Conversely, anti-α-toxin did not neutralize either B. cereus toxin or phospholipase C-α-Toxin and B. cereus phospholipase C and toxin were neutralized by homologous antisera. It was also noted that normal rabbit serum inactivated B. cereus hemolysin without a comparable effect on the hemolytic activity of α-toxin. These data show that B. cereus extracellular products and C. perfringens α-toxin are antigenically distinct.

**DISCUSSION**

The nutritional requirements of B. cereus are not fastidious (1, 16). Growth media composed of amino acids and salts are usually adequate for the growth of the microorganism. However, most media seem to be inadequate for toxin synthesis, regardless of chemical composition. The consistent production of toxin by B. cereus in fresh beef infusion probably reflects the activity of an unidentified nutritional factor(s) which is peculiar to fresh beef.

B. cereus is not a notable mammalian pathogen, although it has been incriminated as the etiologic agent in outbreaks of food-poisoning (11, 12), septicemia (4), bronchopneumonia (27), and bacteremia (5). The culture filtrates of B. cereus are hemolytic for erythrocytes from a variety of species, lethal for laboratory animals, and also possess phospholipase C activity. These composite properties of B. cereus filtrates are comparable to those of α-toxin synthesized by the anaerobic pathogen, C. perfringens.

A superficial comparison of the biological properties of B. cereus filtrates with those of α-toxin led Chu (3) to suggest that the lethality of the former was associated with a phospholipase. This contention that one molecular species was responsible for the lethal and phospholipase activity of B. cereus filtrates was re-examined and found to be incorrect. This conclusion was reached on the basis of the following observations. (i) The synthesis of B. cereus phospholipase C was inhibited by the incorporation of methanol into the growth menstruum, without a comparable effect on toxin synthesis. The action of the solvent was shown to be at the level of synthesis, since dialysis of preformed B. cereus products against 30% methanol did not alter the activity of the filtrates. (ii) Lethal toxin, hemolysin, and phospholipase C appeared concurrently in the growth menstruum, but disappeared at differential rates. (iii) The B. cereus products showed differential susceptibility to temperature, pH, and trypsin. (iv) Gel-filtration data provided conclusive evidence that each activity of B. cereus filtrates was catalyzed by a specific protein. Although the first three observations did not clearly differentiate between different proteins with specific catalytic properties and one protein molecule which possesses multicatalytic sites, they supplemented the gel-filtration data which definitely established the individual nature of the extracellular products of B. cereus.

The phospholipase synthesized by B. cereus was shown to be a phospholipase C by the demonstration that its enzymatic degradation products of a lecithin substrate are diglyceride and phosphorylcholine. A partially purified enzyme preparation was shown to be neither lethal nor hemolytic. This fact would argue against a contribution by phospholipase C to the lethal properties of B. cereus filtrates, as has been suggested by Slein and Logan (26). Although it is nonlethal, B. cereus phospholipase C and the lethal α-toxin of C. perfringens reduce lecithin to comparable degradation products. Common degradation products would indicate that both the α-toxin and B. cereus phospholipase C have a common point of attack on lecithin, although the requirements for the activity of each enzyme are different. The in vitro phospholipase C activity of C. perfringens α-toxin is dependent on

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**TABLE 5. Effect of diethylenetriamine pentaacetic acid (DTPA) in vivo on the lethal activities of Bacillus cereus toxin and Clostridium perfringens α-toxin**

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Toxin</th>
<th>mg dose</th>
<th>Mortality</th>
<th>Protection</th>
</tr>
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<tr>
<td>DTPA</td>
<td>B. cereus toxin</td>
<td>0.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>DTPA</td>
<td>(iv)b</td>
<td>0.01</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA</td>
<td>C. perfringens α-toxin (iv)</td>
<td>0.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>DTPA</td>
<td>C. perfringens α-toxin (iv)</td>
<td>0.025</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a Mice were injected three times with 1.0 mg of DPTA intraperitoneally at 1-hr intervals before challenge with B. cereus toxin or C. perfringens α-toxin. Animals injected with saline rather than DTPA served as controls.

b Intravenous.
c Intraperitoneal.
cations, whereas no cation requirement for the activity of *B. cereus* phospholipase C was found. The activities of *B. cereus* hemolysin and toxin were also found to be independent of metal ions. The pathogenicity of *C. perfringens* is undoubtedly related to its capacity to synthesize α-toxin. The biological activities of *B. cereus* culture filtrates mimic those of α-toxin, yet the microorganism is not an overt pathogen. This rather paradoxical situation represents a fruitful area for investigation and speculation. A detailed biochemical and immunological study directed at uncovering the subtle differences in microbial products which appear to be biologically similar may provide some insight into the fundamental mechanisms of virulence or lack thereof.

**Acknowledgments**

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**Literature Cited**


