SEPARATION OF THE TOXIN OF *BACILLUS CEREUS* INTO TWO COMPONENTS AND NONIDENTITY OF THE TOXIN WITH PHOSPHOLIPASE

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ABSTRACT

MOLNAR, DOROTHY M. (U.S. Army Chemical Corps, Fort Detrick, Frederick, Md.). Separation of the toxin of *Bacillus cereus* into two components and nonidentity of the toxin with phospholipase. J. Bacteriol. **84**:147–153. 1962—*Bacillus cereus* produced toxin in a Casamino acids medium without added serum or other protein. The toxin was separated into two components by adsorption on columns of calcium phosphate gel followed by elution with phosphate buffer (pH 7.5). The component eluted first has been called factor I and the component eluted later factor II. When tested alone each component was relatively nontoxic, but when combined they formed a toxic mixture as evidenced by skin reactions in guinea pigs and tests for lethal effects in mice.

The phospholipase activity, determined by the egg-yolk reaction, was found in the fraction containing factor I. However, using columns of alumina-C_γ gel the phospholipase activity was found in the fraction containing factor II. This suggests that the phospholipase is not the same chemical entity as either factor I or II. The following are further evidence for the nonidentity of the toxin and phospholipase: (i) differential precipitation of the two activities by $(NH_4)_2SO_4$; (ii) differential neutralization by various antisera; and (iii) differential inhibition with ethanol.

Chu (1949) described the toxic reactions of *Bacillus cereus* and linked the toxicity to the lecithinase and hemolytic activity on the basis of the following evidence. (i) Immune sera neutralized the lecithinase activity on free and bound phospholipids as well as the hemolytic, dermonecrotic, and lethal activities. (ii) The fraction, precipitated between one-third and two-thirds saturation with $(NH_4)_2SO_4$, contained the lecithinase, hemolytic, and toxic activities.

On the other hand, the toxicity of other species of bacilli has been dissociated from the phospholipase activity. Evans and Wardlaw (1952) found that the fraction of culture filtrates of *B. subtilis* when precipitated with 50% $(NH_4)_2SO_4$ was hemolytic to the red blood cells of a number of species, dermonecrotic in rabbits, and lethal to mice, but did not give the egg-yolk reaction for lecithinase. Fresh normal sera inhibited the hemolysis, dermonecrosis, and lethal effects. Attempts to produce antisera in rabbits, guinea pigs, and rats were unsuccessful.

Zwartouw and Smith (1956) found that filtrates of the NP strain of *B. anthracis* hydrolyzed the phospholipid in egg-yolk broth but not free egg lecithins or cephalins. Their preparations did not hemolyze guinea pig red blood cells and did not produce a skin reaction in guinea pigs. The phospholipase was not inhibited by antiserum to the Sterne strain.

Costlow (1958) obtained fractions of *B. anthracis* culture filtrates by precipitation with $(NH_4)_2SO_4$. These fractions gave a typical reaction in egg-yolk broth, hydrolyzed free lecithins, hemolyzed sheep and rabbit red blood cells, but were nontoxic to mice and guinea pigs.

This report shows that the marked difference in toxicity of filtrates of *B. cereus* and *B. anthracis* is not owing to the potent phospholipase present in filtrates of *B. cereus*. Furthermore, it describes the separation of the toxin of *B. cereus* into two components, neither one associated with the phospholipase activity.

MATERIALS AND METHODS

Organisms. In the majority of experiments, strain 6464 of *B. cereus* was used, but strains 9139 and 7004 were also shown to produce toxin. Spore suspensions of each strain were prepared for use as inocula and were stored at 5 C.

Production of toxin. The Casamino acids medium of Thorne and Belton (1957) was prepared as described by Thorne, Molnar, and Strange

(1960). Cultures were grown statically at 37 C in either 3-liter Fernbach flasks containing 500 ml of medium or in 250-ml Erlenmeyer flasks containing 50 ml of medium. Spore inocula were used in the amounts given for individual experiments. Usually the $NaHCO_3$ was added 4 hr after inoculation, and the time of incubation varied for individual experiments. After incubation, the cultures were centrifuged at 5 C, and the supernatant liquid was decanted and sterilized by passage through ultrafine fritted-glass filters.

Isolation of crude toxin. Culture filtrates (3 to 5 liters) were saturated with $(NH_4)_2SO_4$ and left at −10 C for 1 to 3 days. The precipitates were collected by filtration through Schleicher and Schuell paper (no. 602) on a Büchner funnel at 5 C and extracted by macerating the paper in about 200 ml of 0.05 M tris(hydroxymethyl)-aminomethane (tris) buffer (pH 8.0). The paper debris was removed by slow-speed centrifugation or by filtration through Whatman no. 1 paper.

Assay of antigen. The agar diffusion method of Thorne and Belton (1957) was used for determining antigens in culture filtrates and in purified preparations. Titers are expressed as arbitrary units; a solution of antigen that just gave a visible precipitation line with antiserum in an Ouchterlony plate contained 1 unit per ml.

Skin test for toxin. The skin test in guinea pigs for determining toxicity was performed as described by Thorne et al. (1960). However, instead of the edematous reaction observed with anthrax toxin, potent preparations of *B. cereus* produced a marked inflammatory reaction, which became necrotic after 24 hr.

Lethality test for toxin. Usually, groups of three to five mice were injected intravenously or intraperitoneally with 0.5-ml volumes of serial dilutions of toxin preparations. Mice injected intravenously with lethal concentrations usually died within 5 to 10 min, whereas mice injected intraperitoneally with the same concentrations usually died in 1 to 2 hr. Mice were observed for 4 days, and the results were reported as the number of deaths per number of mice injected. Toxin titers refer to the highest dilution killing 50% or more of each test group.

Test for phospholipase. In the lecitho-vitellin (LV) test for phospholipase activity, egg-yolk broth was observed for the formation of an opalescent suspension or a flocculent curd. Small tubes containing 2 ml of egg-yolk broth (about 5% fresh egg yolk in nutrient broth) were inoculated with 0.2-ml volumes of serial dilutions of the sample and were incubated for 4 hr at room temperature. LV titers are expressed as the reciprocal of the highest dilution giving a positive reaction.

Determination of protein. Total protein was estimated by the method of Lowry et al. (1951).

RESULTS

Unsuccessful attempts to separate toxin and phospholipase. The toxin of *B. anthracis* was separated into two components by passing culture supernatant fluid containing no serum through sintered-glass filters (Thorne et al., 1960). Similar efforts to separate the toxin and phospholipase activities of *B. cereus* were not successful. The same titers were obtained in all tests whether serum was added before or after filtration, or not added at all. In some experiments, addition of serum during growth increased the yields of both toxin and phospholipase.

Varying the concentration of $NaHCO_3$ in the medium failed to differentiate between the production of toxin and phospholipase. In the absence of added $NaHCO_3$, neither toxin nor phospholipase was detected, whereas the ratio of the two activities was not changed significantly at 0.3, 0.6, and 0.9% concentrations of $NaHCO_3$.

When cells were grown in shaken cultures, neither toxin nor phospholipase was detected from 16 to 48 hr. When cells were grown in static cultures, the titers of both activities were maximal from 16 to 72 hr. At 8 hr neither was detectable, whereas after 72 hr the titers of both had decreased.

Heating the culture filtrate at 100 C destroyed toxin and phospholipase activity at parallel rates, indicating that these activities might be properties of the same chemical entity.

Fractionation with $(NH_4)_2SO_4$. A solution of crude toxin, prepared as described in Materials and Methods, was brought to 30% saturation with $(NH_4)_2SO_4$ by the addition of solid salt and left at 5 C for 16 hr. The precipitate that formed had negligible activity, was removed by centrifugation, and was discarded. More solid salt was added to the supernatant solution and fractions were collected at 50, 60, and 80% saturation with $(NH_4)_2SO_4$. The precipitate that formed at each

TABLE 1. *Fractionation of a culture filtrate by precipitation with* $(NH_4)_2SO_4$

Fraction	Toxin (guinea pig skin test)				Phospholipase (LV test)*			Toxin titer/ phospholipase titer
	Volume	Titer	Total	Recovery	Titer	Total	Recovery	
	ml	*units/ml*	*units*	%	*units/ml*	*units*	%	
Culture filtrate	750	64	48,000		8	6,000		8/1
$(NH_4)_2SO_4$ saturation								
0 to 100%	41	512	21,000	43	128	5,600	87	4/1
30 to 50%	8	1,024	8,000	17	512	4,100	68	2/1
50 to 60%	8	1,024	8,000	17	128	1,000	17	8/1
60 to 80%	7	256	2,000	4.2	8	50	0.8	32/1

* LV test: lecitho-vitellin reaction in egg-yolk broth.

step was collected by centrifugation and dissolved in 0.05 M tris buffer (pH 8.0). All three fractions contained both toxin and phospholipase (Table 1). However, the ratio of toxin to phospholipase increased with increasing concentration of $(NH_4)_2SO_4$. If the toxicity and phospholipase activity were owing to the same molecule, the ratio of the two activities in different fractions should remain constant.

Neutralization of the toxic and phospholipase activities. Specific antisera were prepared by injecting rabbits on alternate days with increasing doses of spores of *B. cereus*. Antisera 1 and 4 neutralized the phospholipase activity, the toxicity in the skin, and tests for lethal effects (Table 2). Antiserum 5 failed to inhibit the lethal effect completely, but neutralized the toxicity in the skin test and the phospholipase activity. Antiserum DH-1-5C, prepared in a horse by injecting spores of the Sterne strain of *B. anthracis*, did not inhibit any of the activities of *B. cereus* tested.

Although toxicity of *B. cereus* filtrates has not been correlated with a specific line of precipitation in the agar diffusion test, the lines of precipitation formed by toxic filtrates were neutralized by all of the homologous antisera, but not by any of the normal sera or the heterologous antiserum.

Effect of ethanol on toxic and phospholipase activities. Partially purified preparations of toxin were dialyzed against 30 and 40% ethanol (v/v) as indicated in Table 3. The phospholipase activity of the preparations was not precipitated or inactivated by either concentration of ethanol. On the other hand, most of the toxicity was destroyed by dialysis against either concentration. The inactivation was not owing to the separation of the toxin into two component parts, since recombination of the precipitates with the final supernatant fluid did not restore toxicity.

Separation of toxin into two components by column chromatography. To confirm the separation of the toxin and phospholipase activities, columns of $Ca_3(PO_4)_2$ gel (16 mg/ml) in 10-ml Mohr pipettes were used. To increase the flow of solvent through the packed columns, acid-washed Celite 545 (does not adsorb toxin) was mixed with the adsorbent in a weight ratio of about 1 part gel to 10 parts Celite. This slurry was added to the pipettes and washed with water. Then each column was charged with 2 ml of a crude toxin preparation, washed with 10 ml of water, and eluted with about 20 ml of 0.1 M phosphate buffer (pH 7.5). Fractions collected in 2-ml volumes at 30-min intervals were tested for phospholipase and toxic activities.

All the toxic activity was adsorbed on the column and not washed through with water, as some of the phospholipase activity was (Table 4). The first fractions eluted with phosphate buffer (tubes 6 to 8) contained phospholipase but no toxin. Tubes 9 to 12 contained a low level of toxin and practically no phospholipase. The total recovery of phospholipase was over 100%, but the recovery of toxin as determined in the skin test was only 6% and nil in the test for lethal effect. Therefore, the fractions were pooled into two groups, which were tested singly and in combination. When tested alone both groups 1 (tubes 6 to 8) and 2 (tubes 9 to 15) had low titers of toxin in the skin test and were not lethal at a dilution of 1:4. However, a mixture of equal

TABLE 2. *Neutralization of toxin and phospholipase by antisera*

Test solution*	Phospholipase/LV test	Toxin activity		
		Guinea pig skin test	Test for lethal effect in mice†	
			No. dead/no. mice	Time to death
	units/ml	*units/ml*		
Toxin +				
Gelatin phosphate	16	16	10/10	<10 min
Normal serum 4	16	16	5/5	<10 min
Antiserum 4	0	0	0/5	—
Antiserum 1	0	0	0/5	—
Antiserum 5	0	0	3/5	<18 hr
Normal horse serum	16	16	5/5	<10 min
Antiserum DH-1-5C	16	16	5/5	<10 min

* Twofold serial dilutions were made in gelatin phosphate after mixing initially 2 ml of culture filtrate, 1 ml of gelatin phosphate, and 1 ml of test solution. Antisera 1, 4, and 5 were prepared in rabbits against *Bacillus cereus* spores, and antiserum DH-1-5C was prepared in a horse against spores of *B. anthracis*, strain Sterne. Gelatin phosphate = 0.5% (w/v) gelatin in 0.02 M phosphate buffer (pH 8.0).

† Filtrate (0.25 ml) injected intravenously.

parts of the two groups had a toxin titer of 64 in the skin test and killed mice at a dilution of 1:16. On the other hand, the phospholipase titer of the mixture was the same as that of group 1 alone. These results show that the toxin of *B. cereus* can be separated into two components that act synergistically to produce a toxic reaction, but not the phospholipase reaction. Hereafter, the first component of toxin eluted from columns of $Ca_3(PO_4)_2$ gel is called factor I and the other component, eluted later, is called factor II.

Using a larger column (7 by 3/4 in.) of Al-C_γ gel (20 mg/ml) and Celite 545, toxin was also separated into two components (Table 5). This column was charged with 6 ml of another crude preparation of toxin, washed with water, and eluted with 0.1 M phosphate buffer (pH 7.5). Fractions (1 ml) were collected at 30-min intervals and pooled into groups on the basis of lines of precipitation formed against *B. cereus* antiserum. When tested alone, groups M3 (tubes 41 to 56) and M6 (tubes 79 to 90) were nontoxic; when tested together, they formed a synergistic mixture that was edematous in guinea pigs and lethal to mice. Evidence that fractions M3 and M6 were identical with factors I and II, respectively, was the formation of toxic mixtures with M3 and factor II and with M6 and factor I, whereas mixtures of M3 and factor I and of M6 and factor II were nontoxic. From the Al-C_γ column, the phospholipase activity was eluted along with factor II (fraction M6) rather than with factor I as observed with columns of $Ca_3(PO_4)_2$ gel. This would indicate that phospholipase is not one of the components of toxin.

Inactivation of toxin components. With other preparations of factors I and II, the results in Table 6 show that the toxicity of a mixture of them is neutralized by antiserum prepared against spores of *B. cereus* or against a crude toxin preparation. Heating either factor prior

TABLE 3. *Effect of ethanol on the toxic and phospholipase activities of crude preparations*

Fraction	Toxin activity (units/ml)		Phospholipase activity (units/ml)	
	Guinea pig skin test		LV test	
	C*	D	C	D
Before treatment	512	1024	512	256
Ethanol (30%) + supernatant fluid	128	64	512	256
Ethanol (30%) + precipitate	8	8	32	0
Ethanol (40%) + supernatant fluid	64	32	512	256
Ethanol (40%) + precipitate	0	0	8	8
Precipitates (30% + 40%)	8	8		
Precipitates (30% + 40%) + supernatant fluid	64	64		

* C and D were preparations that were precipitated with $(NH_4)_2SO_4$ at 30 to 50% and 50 to 60% saturation, respectively. Each preparation was dialyzed against 30% ethanol in 0.2 M phosphate (pH 7) at −10 C for 16 hr, centrifuged at −5 C, and the precipitate dissolved in 0.05 M phosphate (pH 7.5). The supernatant fluid was then dialyzed against 40% ethanol in 0.05 M phosphate (pH 7) and treated as above.

TABLE 4. *Separation of toxin into two components by column chromatography on $Ca_3(PO_4)_2$ gel and Celite 545*

Tube no.	Eluent*	Phospholipase	Toxin activity	
		LV test	Guinea pig skin test	Test for lethal effect in mice†
		units/ml	*units/ml*	*no. dead/ no. injected*
1	H_2O	0	0	0/3
2	H_2O	16	0	0/3
3	H_2O	0	0	0/3
4	H_2O	0	0	0/3
5	H_2O	0	0	0/3
6	Buffer	16	0	0/3
7	Buffer	64	0	0/3
8	Buffer	4	0	0/3
9	Buffer	4	4	0/3
10	Buffer	0	4	0/3
11	Buffer	0	4	0/3
12	Buffer	0	4	0/3
13	Buffer	0	0	0/3
14	Buffer	0	0	0/3
15	Buffer	0	0	0/3
6 to 8	Factor I	32	4	0/3
9 to 15	Factor II	0	8	0/3
6 to 8 + 9 to 15		32	64	3/3

* A 10-ml column of $Ca_3(PO_4)_2$ gel and Celite 545 was charged with 2 ml of a crude preparation of toxin, washed with water (tubes 1 to 5), and eluted with 0.1 M PO_4, pH 7.5 (tubes 6 to 15); fractions of 2-ml volume were collected every 30 min. Crude toxin had the following titers: LV test, 128; skin test, 256; and lethality, 128.

† Each mouse was injected ip with 0.5 ml of a 1:4 dilution of eluate.

to mixing with the other factor reduced the toxicity of the resultant mixture. Toxic mixtures were not formed when either factor was replaced by autoclaved medium or autoclaved culture filtrate.

Further evidence that neither component of the toxin is phospholipase was also shown. Fraction Y5, which was collected from a column of Al-C_γ gel and Celite and which contained a large amount of phospholipase along with a trace of factor II, did not increase the toxicity of a mixture of factors I and II. When mixed with factor I alone, fraction Y5 contained enough factor II to give a skin reaction but not to kill mice.

Evidence for protein nature of toxin components. Solutions of factor I usually produced two or more lines of precipitation with *B. cereus* antiserum, whereas solutions of factor II produced one or two lines. When placed in adjacent reservoirs on agar diffusion plates, the line or lines formed by factor II crossed the lines produced by factor I. Both components are probably protein, since both were inactivated by heat, precipitated by $(NH_4)_2SO_4$, and were nondialyzable. However, the individual components were much less stable to dialysis and storage than the crude toxin preparations. This instability and limited supply have precluded additional tests on their chemical composition.

Effect of limiting amounts of either component on skin-test titer. One of the difficulties involved in separating and purifying the two components of toxin is exemplified in the results presented in Table 7. Two different preparations of each component were used in two separate experiments; negligible titers were obtained when the

TABLE 5. *Separation of toxin into two components by column chromatography on Al-C_γ gel and Celite 545**

Groups†	Phospholipase	Toxin activity	
	LV test	Guinea pig skin test	Test for lethal effect in mice‡
	units/ml	*units/ml*	*no. dead/ no. mice*
M3	0	0	0/3
M6	8	0	0/3
M3 + M6	8	32	3/3
M3 + Factor I		4	
M3 + Factor II	0	16	
M6 + Factor I	32	32	3/3
M6 + Factor II		4	
Factor I	16	0	0/3
Factor II	0	4	0/3
Factor I + Factor II		64	3/3

* A column (7 by ¾ in.) of Al-C_γ gel and Celite was charged with 6 ml of a crude preparation of toxin, washed with water, and eluted with 0.1 M PO_4 (pH 7.5).

† Factors I and II were obtained from columns of $Ca_3(PO_4)_2$ gel.

‡ Eluate (0.125 ml) injected intraperitoneally.

TABLE 6. *Inactivation of toxin components*

Test material*	Phospholipase	Toxin activity	
	LV test	Guinea pig skin test	Test for lethal effect in mice†
	units/ml	*units/ml*	*no. dead/ no. injected*
Factor I	8	1	0/3
Factor II	0	1	0/3
Fraction Y5	32	1	0/3
Factor I and factor II	8	64	3/3
Factor I + factor II + spore antiserum		4	0/3
Factor I + factor II + crude toxin antiserum		4	0/3
Factor I + factor II + fraction Y5		64	3/3
Factor I + fraction Y5		16	0/3
Factor I + heated factor II		8	0/3
Factor II + fraction Y5		4	0/3
Factor II + heated factor I		8	0/3

* Factors I and II were obtained from columns of $Ca_3(PO_4)_2$ gel + Celite 545. Fraction Y5 was the last fraction collected from a column of Al-Cγ gel + Celite 545. Heated factors were held in boiling water for 10 min.

† Material (0.125 ml) injected intraperitoneally.

components were diluted singly in gelatin phosphate. When equal volumes of the two were mixed and diluted in gelatin phosphate, the titer was 64 in one experiment and 16 in the other. When one factor was diluted serially in a constant amount of the other (below the level giving a skin reaction), the titer was increased to 128 and 256. On the other hand, neither autoclaved medium nor the supernatant fluid from a preparation saturated with $(NH_4)_2SO_4$ increased the titers of mixtures of factors I and II in gelatin phosphate. Evidently, the titers obtained in the skin test are markedly influenced by limiting amounts of either factor I or II, and not by some other component of the medium or preparation.

DISCUSSION

Culture filtrates of *B. cereus*, injected intradermally in guinea pigs, produced a marked inflammatory reaction, which became necrotic after 24 hr. At dilutions near the end point, the skin reaction resembled the edematous reaction produced on injection of culture filtrates of *B. anthracis*. *B. cereus* filtrates killed mice in 5 to 10 min after intravenous injections of 0.5-ml volumes of 1:4 to 1:8 dilutions. With *B. anthracis*, 1 ml of undiluted filtrate was usually required to kill mice in 24 to 72 hr.

TABLE 7. *Effect of limiting amounts of either component of toxin on skin-test titer*

Test system	Toxin activity: Skin-test titer	
	Expt 1	Expt 2
Factor I diluted in gelatin phosphate	4	2
Factor II diluted in gelatin phosphate	2	2
Factors I + II diluted in gelatin phosphate	64	16
Factors I + II diluted in gelatin phosphate + CA medium*	64	
Factors I + II diluted in gelatin phosphate + supernatant fluid from 100% $(NH_4)_2SO_4$†	64	
Factor I diluted in II (constant level of 1:8)	128	128
Factor II diluted in I (constant level of 1:8)	256	128

* CA medium was the same as that used for growth but was filtered without being inoculated.

† Supernatant fluid from 100% $(NH_4)_2SO_4$ was that obtained after filtering a culture fluid which had been saturated with $(NH_4)_2SO_4$.

Chu (1949) linked the toxicity of *B. cereus* with its phospholipase activity, although he pointed out the impure nature of his preparations. Zwartouw and Smith (1956) and Costlow (1958) reported that the toxin of *B. anthracis* was not associated with phospholipase. Nevertheless, *B. anthracis* is not an active producer of phospholipase, and the difference in toxicity between the two organisms might be owing to this difference in phospholipase activity. At first, our results supported Chu's conclusion that toxicity and phospholipase activity were identical, since variation in growth conditions and time of incubation failed to distinguish the toxic and phospholipase activities. However, the differential precipitation of the two activities with $(NH_4)_2SO_4$, the differential neutralization by various antisera, and the differential inhibition by ethanol showed that the toxicity and phospholipase activity are

owing to different molecules. Furthermore, the separation of the toxin into two components by column chromatography on $Ca_3(PO_4)_2$ gel and Al-C_γ gel offered evidence of the nonidentity of phospholipase with either component of the toxin. Further work is required to explain the marked difference in toxicity between *B. cereus* and *B. anthracis*.

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