PARTIAL PURIFICATION AND PROPERTIES OF TWO PHOSPHOLIPASES OF *BACILLUS CEREUS*

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ABSTRACT

SLEIN, MILTON W. (U.S. Army Chemical Corps Biological Laboratories, Fort Detrick, Frederick, Md.) AND GERALD F. LOGAN, JR. Partial purification and properties of two phospholipases of *Bacillus cereus*. J. Bacteriol. **85**:369–381. 1963.—Culture filtrates of *Bacillus cereus* contain a phosphatasemia factor (PF) that markedly increases blood alkaline phosphatase after intravenous injection into animals, and that releases alkaline phosphatase from epiphyseal bone slices in vitro. Fractionation of culture filtrates of *B. cereus* with *N*,*N*′-diethylaminoethyl cellulose results in the separation of two phospholipases, one that has PF activity and one that inhibits PF activity in vitro. Growth of shaken cultures favors accumulation of the inhibitor, whereas static cultures yield more PF. Lethality for mice and hemolysin activity do not appear to be associated with the phospholipase that inhibits PF. The relationship of the lethal and hemolysin factors to the phospholipase that produces phosphatasemia is not clear. The effects of heat, trypsin, lecithin, and antiserum on the phospholipases are reported. The intravenous injection of relatively large amounts of the purified PF resulted in the depletion of bone alkaline phosphatase.

Although the production of phosphatasemia factor (PF) in culture filtrates of certain bacilli appeared to be related to that of phospholipase C (lecithinase), previously reported evidence supported the conclusion that the two activities were not identical (Slein and Logan, 1962*a*). [Phospholipase C is used to designate the enzyme that splits lecithin into a diglyceride and acid-soluble phosphorylcholine, according to the nomenclature given by Hayaishi (1955). Unfortunately, the nomenclature of phospholipases has been variable, and the same enzyme has also been called phospholipase D (Deuel, 1955) and lipophosphodiesterase I (Schmidt and Laskowski, 1961).] More recent findings show that this interpretation probably was confused by the presence of more than one phospholipase, as well as an inhibitor of PF activity (Slein and Logan, 1962*b*). Results reported in this paper are concerned with the fractionation of culture filtrates of *Bacillus cereus*, some properties of the fractions, and further evidence that bone epiphyses contribute to the phosphatasemia that results from the intravenous injection of PF into rabbits.

MATERIALS AND METHODS

Culture filtrates and other preparations. Strains 6464 and 7004 of *B. cereus* were originally obtained from the American Type Culture Collection. Cell-free filtrates were prepared after cultures had been grown statically for 24 hr at 37 C, as described previously but without the addition of charcoal (Slein and Logan, 1962*a*). Preparations were concentrated by saturating the culture filtrates with $(NH_4)_2SO_4$ at 5 C. The precipitate was dissolved with distilled water, and was dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (tris), pH 7.5. In most cases, the clear, slightly amber, concentrated material was treated with protamine sulfate to remove nucleic acids and much of the color. An excess of protamine was avoided by the careful addition of a 2% solution of protamine sulfate, between centrifugations, to remove the bulk of the precipitate as it formed at room temperature (23 C). When the precipitation is carried out at about 0 C, the protamine itself forms a copious precipitate as it is added to the cold solution. The precipitate of undiluted cooled protamine will redissolve when stirred, whereas the complex of protamine with nucleic acid will not; thus, it is possible to avoid the addition of excess protamine, even in the cold, if this is kept in mind. The preparations were dialyzed again after treatment with protamine.

Phospholipase C assay. The assay procedure was essentially as described by Costlow (1958). Soybean lecithin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and from Mann Research Laboratories, Inc. The reaction mixture (3 ml) contained 100 mg of lecithin, which had been emulsified in a Raytheon sonic oscillator (Hayaishi, 1955). The mixture, buffered with barbital at pH 7.6, was incubated in the presence of 0.01 M $CaCl_2$ for 30 min at 37 C; the reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid. After filtration, acid-soluble phosphorus in 0.1 ml of the filtrate was determined in a final volume of 3.3 ml at 660 mμ with a 1-cm light path. One unit of enzyme activity is defined as an increase in absorbancy of 0.001. To conserve enzyme and substrate, half of the normal reagent volumes were sometimes used, but the specific activity was calculated for a volume of 3 ml. Proportionality was obtained with as much as 300 units of enzyme per 3 ml of reaction mixture.

Bone slice test for PF. The procedure of the test was basically that described previously (Slein and Logan, 1962*a*). Although we used only the proximal tibial and distal femoral epiphyses of Dutch rabbits weighing about 1 kg, other epiphyseal bone of young animals probably could be used. The untrimmed bones may be stored packed in ice for a few days without apparent loss of ability to respond to PF. Just before slicing, the joint was freed from adherent muscle, cartilage, and other tissues. The bone was held firmly in the clamp of a Spencer model 860 sliding microtome. The blade was kept wet with Ringer phosphate solution, and slices were cut approximately 120 μ thick. They were immediately placed in small beakers of cold Ringer phosphate solution in an ice bath. Usually, 49 slices were obtained from the knee-joint epiphyses. The slices were distributed serially among seven beakers, so that each contained seven slices matched with the others. The slices were transferred to manometer flasks, each containing 3 ml of cold Ringer phosphate solution, and the flasks were shaken at about 100 cycle/min for 15 min at 37 C. Initial samples, before incubation at 37 C, were not taken, because the alkaline phosphatase activity in such samples was consistently low. To minimize the presence of floating particles after centrifugation, bits of cotton wool were wound about the tapered ground tips of 1-ml pipettes used for taking 0.6-ml samples from the flasks. The samples were placed in 10 × 100-mm tubes in an ice bath, and 0.3 ml, or less, of PF or other test solution was added to each flask. The flasks were shaken for another 15 min and were sampled again. The samples were chilled, centrifuged, diluted, and tested for alkaline phosphatase, as reported previously (Slein and Logan, 1962*a*).

Since the slice test for PF is an indirect one, it is important to establish that the response measured (alkaline phosphatase release) is proportional to the concentration of factor added, and that the phosphatase activity is not directly inhibited or stimulated after being released from the slices. We have established that PF does not merely activate soluble bone alkaline phosphatase. It has been determined that the effect of inhibitor fractions is not due to the inactivation of bone phosphatase, but rather to the inhibition of the PF-induced release of phosphatase from the slices. Bone alkaline phosphatase activity is directly proportional to the amount of enzyme, over a wide range of activity. Linear proportionality was found to exist from 0 to an absorbancy of at least 1.300 at 395 mμ after incubation of the enzyme for 15 min at 37 C, which was the time used routinely for the measurement of bone alkaline phosphatase. However, measurement of the amount of alkaline phosphatase released from bone slices in 15 min at 37 C is limited to an absorbancy range of about 0 to 0.400. The limit is determined by the number of slices used in each flask. With seven slices of epiphyseal bone, having a blotted moist weight of 50 to 70 mg, the release of alkaline phosphatase, in the limited range just mentioned, is approximately proportional to the amount of PF added. The phosphatase released by PF must be corrected for the spontaneous leakage from the slices, which usually amounts to an absorbancy of 0.080 to 0.120. One unit of PF is defined as the amount that releases sufficient phosphatase from the bone slices in 15 min to produce an absorbancy of 0.100 in the standard phosphatase test.

Lethality tests. The lethal effect of a preparation was determined by injecting the material, diluted with 0.9% NaCl or Ringer phosphate solution, into the tail veins of Swiss mice weighing 15 to 20 g. The smallest amount of solution that caused death within 20 min was defined as a

MLD. Mice that survived for more than 20 min usually did not die at all from the injected material. A MLD usually caused death within 5 to 8 min.

Hemolysins. Hemolytic activity was detected on sheep-blood agar plates, prepared as described by Skerman (1959). Samples (0.1 ml) were allowed to spread in a circular area on the surface of the agar. After 1 or 2 hr at 25 C, the plates were incubated at 37 C, and were examined at intervals for comparison of the rates at which hemolysis occurred.

Paper electrophoresis. Electrophoresis was carried out with 0.05 M barbital buffer (pH 8.6). The enzyme solutions were spotted on three strips of Whatman 3MM paper (3.5 × 30 cm, excluding the submerged portions). The chamber was cooled with circulating water at 7 C, and 500 to 600 v were applied for about 4 hr. A thin section was cut from the edge of each paper strip, to be dried and stained with bromophenol blue for locating proteins (Kunkel and Tiselius, 1951). The rest of the strips were kept at 5 C between sheets of polyethylene, to minimize drying while the control sections were being stained. The unstained strips were then cut into 0.5-in. sections at various distances from the origin, and each section was cut into smaller pieces. These were placed in a Virtis micro homogenizer cup with 1 ml of cold distilled water, and were homogenized for about 2 min. The paper pulp was centrifuged, and residual liquid was squeezed from the precipitate with a glass rod. Electrophoresis was tested at other pH values as low as pH 4.4, but the results only at pH 8.6 will be discussed.

Fractionation on N,N'-diethylaminoethyl (DEAE) cellulose. DEAE cellulose was obtained from Eastman Kodak Co., Rochester, N.Y. A slurry of the material in 0.25 N NaOH was used for preparing a column about 1.2 × 17 cm. The column was washed with NaOH and distilled water, and was equilibrated with tris buffer (pH 8.6). When elution was to be done with step-wise increases in buffer concentration, 0.05 M tris was used for equilibration, and the concentration of tris in the enzyme solution was adjusted to about 0.05 M before placing it on the column. When continuous gradient elution was used, the column was equilibrated with 0.01 M tris, and the enzyme solution was dialyzed against 0.01 M tris before it was placed on the column. Elution was carried out at 23 C with a flow rate of about 2 ml/min. Fractions of 5 or 10 ml were collected. With continuous gradient elution, the concentration of tris was estimated at intervals by taking samples from the mixing chamber for the measurement of absorbancy at 230 mμ. After elution from the column, the absorbancy of fractions at 280 mμ was measured.

When it was necessary to concentrate fractions, they were placed in cellophane dialysis tubing, buried in Carbowax 20-M, at 5 C until the desired decrease in volume had occurred. The tubing was rinsed off with distilled water, and the concentrated solution was dialyzed at 5 C against 0.01 M tris (pH 7.5). Carbowax, a polyethylene glycol, was obtained from the Union Carbide Corp., New York, N.Y.

Other procedures were described previously (Slein and Logan, 1962*a*). In some cases, protein was estimated by the method of Waddell (1956).

Results

Preliminary attempts at fractionation. It was known that PF activity could be concentrated by saturating culture filtrates with $(NH_4)_2SO_4$. The addition of protamine removed much of the pigment from the solutions, as well as nucleic acids, and the specific activities of PF and phospholipase were increased three- to fourfold. Further attempts at purification, by means of $(NH_4)_2SO_4$ or isoelectric precipitation, were not very promising.

Electrophoresis. The first direct evidence for the existence of more than one phospholipase in concentrated partially purified preparations from *B. cereus* 6464 was found when fractions were eluted from paper after electrophoresis. Although the recovery of protein was fair, rather poor recovery of PF and phospholipase C activities was obtained, especially at pH 8.6. However, sufficient material was recovered for a comparison of the activities from various regions of the filter papers. In one test (Table 1), at least two phospholipases were present. Most of the PF activity was associated with a phospholipase that moved toward the anode, whereas the phospholipase with the highest specific activity migrated toward the cathode, and was associated with much less PF activity.

Since at least two phospholipase C moieties were separated by electrophoresis at pH 8.6, we

TABLE 1. *Effect of electrophoresis at pH 8.6 on the phospholipase and phosphatasemia factor (PF) activities of protein concentrated from a culture filtrate of Bacillus cereus 6464*

Position on filter paper	Phospholipase C activity	PF activity
	units/μg	*units/μg*
Anode 2	11.2	13.9
Anode 1	18.4	26.6
Origin	7.7	7.8
Cathode	43.8	9.3

decided to try chromatography at the same pH on a DEAE cellulose anion-exchange column.

Fractionation with DEAE cellulose. Residual color that was present in solutions after treatment with $(NH_4)_2SO_4$ and protamine was removed, since the pigment remained stationary at the top of the DEAE cellulose column during elution with tris (pH 8.6). All fractions were clear and colorless. When elution was step-wise, starting with 0.05 M buffer, material with the highest absorbancy at 280 mμ came through the column without being adsorbed. This material probably corresponded to that which moved toward the cathode during electrophoresis. In one procedure, 55 fractions of about 10 ml each were obtained with six concentrations of tris ranging from 0.05 to 1.0 M. Various activities were measured in 15 of the fractions after dialysis against 0.01 M tris (pH 7.5). Data are given in Table 2 for the six principal fractions that had peak absorbancies at 280 mμ with the six concentrations of tris used. Fraction 2, which was not adsorbed to the column, had the highest phospholipase C activity, but it had no PF activity in vitro or in vivo. Fraction 23, which was eluted by 0.4 M tris, contained phospholipase C with about 40% of the specific activity of that in fraction 2, but it had the highest PF activity. The separation of at least two phospholipase activities is obvious from these data.

The fact that hemolysin activity was not detected in fraction 2, but was in fraction 13, indicated that it was not associated with the phospholipase C activity of fraction 2. Hemolysin was also present in four of the other five fractions included in Table 2. Since the hemolysin activity in fraction 13 appeared to be stronger than that in fraction 23, it seems likely that the phospholipase C activity of fraction 23 was not responsible for hemolysin activity. These results are in agreement with the conclusions of Ottolenghi, Gollub, and Ulin (1961), that hemolysin activity could be separated from phospholipase activity.

The original preparation used for fractionation in Table 2 contained approximately one MLD per 10 μg of protein. Although the phospholipase C activity was increased about two- to fourfold in fractions 23 and 2, and the PF activity was increased about fourfold in fraction 23, no lethal factor activity was detected in these, nor in any of the other 13 fractions tested. Thus, it seems unlikely that mouse lethal activity is identical with either of the phospholipases of fractions 2 and 23. The amounts of protein available for lethality tests with the other fractions (about 6 to 18 μg per mouse) were sufficient only to indicate that lethal factor had not been highly concentrated in any of the fractions tested.

It was discovered that fraction 2 not only did not have PF activity, but that it was strongly inhibitory to added PF in the bone-slice test (Table 3). Approximately 4 μg of fraction 2 protein had no detectable PF activity. Only 0.5 μg was required to inhibit PF activity about 70%, whereas 1 μg inhibited the PF completely. Heating the inhibitor for 5 min at 100 C completely inactivated it. Of the 15 fractions tested, 1, 10, 13, and 20 also had no PF activity but only fraction 2, which had the highest specific

TABLE 2. *Fractionation of a partially purified phospholipase of Bacillus cereus 6464 by elution from a DEAE cellulose column with step-wise increases of tris buffer, pH 8.6*

Molarity of tris	Fraction no.	Phospholipase C activity	PF activity		Hemolysin
			In vitro	In vivo*	
		units/μg	*units/μg*	*units/μg*	
0.05	2	124.0	0	0	−
0.2	13	0.8	0	0	+
0.4	23	49.5	173.0	719	+
0.6	33	7.3	10.8	67	+
0.8	44	5.4	8.3	30	+
1.0	53	0.7	1.0	0	−
	Original material	28.0	45.0	149	+

* Increase in blood alkaline phosphatase activity 1 hr after the intravenous injection of 15 μg of protein into a rabbit.

phospholipase C activity, was found to inhibit PF activity.

Figure 1 gives the elution pattern obtained with a buffer concentration gradient that approached linearity; each fraction contained about 5 ml. Only the PF and phospholipase C activities are presented, since the 280 mμ absorbancy pattern of the fractions did not reveal the positions of the active fractions. Since the units of activity are arbitrary and not directly related, the similarity of the specific activities of PF and phospholipase C in fractions 52 to 80 is coincidental. However, the similar distribution in these fractions indicates the identity of the two activities. The concentration of Ca^{++} (0.01 M) used for the measurement of phospholipase C activities of the fractions in Fig. 1 was twice that used previously. Only 0.005 M Ca^{++} was used for phospholipase C activity determinations with another lecithin preparation at the time that the data for Table 2 were obtained; this resulted in relatively lower values for phospholipase.

As with the step-wise gradient, the fractions eluted by the lower concentrations of buffer contained phospholipase activity that was associated with an inhibitor for PF. The PF and phospholipase C present in fraction 62 had the highest activities we have so far obtained. A PF

TABLE 3. *Inhibition of phosphatasemia factor (PF) activity by the protein in fraction 2 of Table 2*

Additions to bone slices	Alkaline phosphatase released
Fraction 2 (4.0 μg)	0
PF (0.2 μg)	312
PF (0.2 μg) + fraction 2 (0.5 μg)	90
PF (0.2 μg) + fraction 2 (1.0 μg)	0
PF (0.2 μg) + heated fraction 2 (2.0 μg)	332

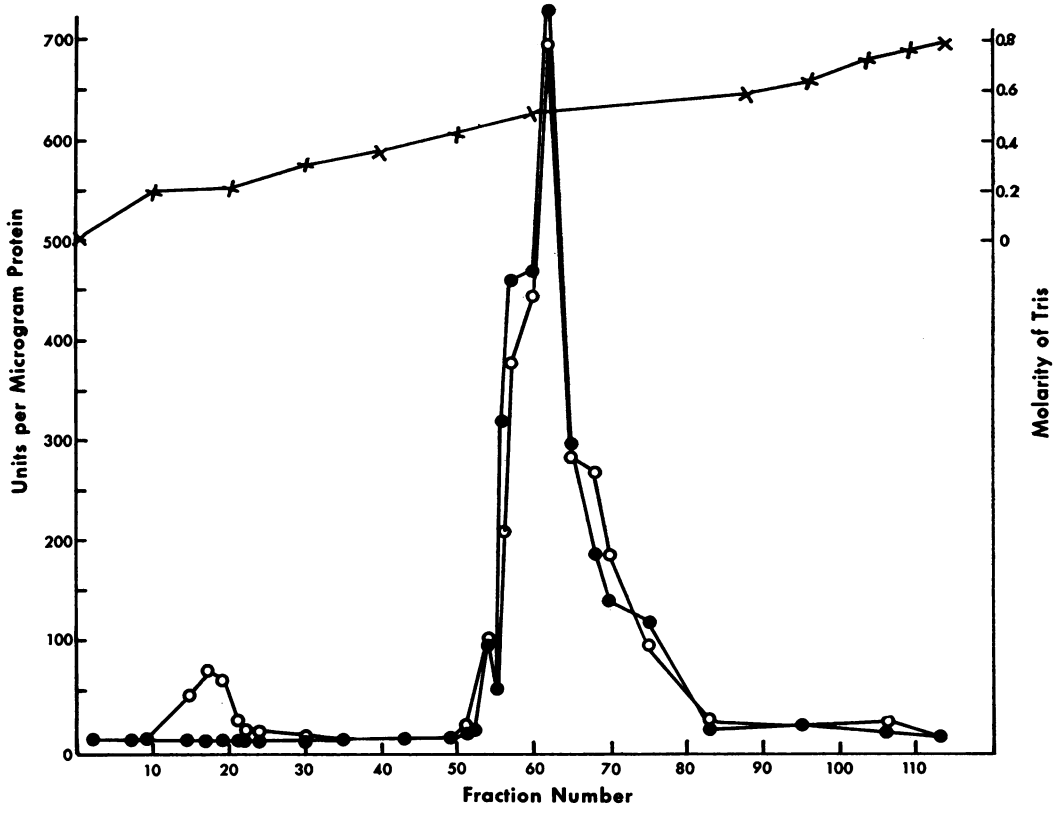

FIG. 1. *Distribution of phosphatasemia factor (PF) and phospholipase C activities in fractions obtained by the elution of a partially purified preparation of PF of Bacillus cereus 6464 from a DEAE cellulose column with increasing concentrations of tris buffer (pH 8.6).* ×, *molarity of tris;* ○, *phospholipase activity;* ●, *PF activity measured with bone slices.*

activity of 750 units/μg in the bone-slice test means that 0.005 μg of protein was sufficient to result in an absorbancy of 0.375, which is a very significant value at the upper limits for proportionality with the method.

To test for lethal factor and hemolysin activity in groups of fractions obtained by the linear gradient elution, several fractions were combined, concentrated, and dialyzed (Table 4). Sample A contained fractions 13 to 20; B contained 30, 34, 35, 36, 43, 48, 49, and 50; C contained 56 to 68; and D contained 80 to 114. Although not all fractions were included, the four samples represented various areas of the gradient and contained material from 64 of the 114 fractions. The 64 fractions represented about 20% of the total protein that was placed on the column for elution. Only 8 μg of protein of the original material was required to kill mice, but mice survived the injection of 20 to 51 μg of protein of samples A, B, and C. A combination of 72 μg of protein prepared from the same three samples also did not kill mice. However, approximately 15 μg of protein in sample D were lethal. These results indicate that PF and its associated phospholipase C are not responsible for the lethality, since sample D contained 10 to 20 times less PF and phospholipase activities than did sample C. Although twice as much protein in sample D was required for lethality as in the original material, it is possible that the lethal factor was concentrated in only a few of the 35 fractions that were combined in the preparation of sample D.

TABLE 4. *Activities of several fractions obtained by the continuous gradient elution of a partially purified Bacillus cereus 6464 phospholipase from DEAE cellulose*

Fraction no.*	PF activity in vitro	Phospholipase C activity	MLD	Hemolysin†
	units/μg	*units/μg*	*μg*	
Original material	66	49	8	+
A	0	28.1	>51‡	−
B	1.6	4.4	>29‡	−
C	430	380	>20‡	−
D	20.6	36.5	15	+

* Several fractions obtained by the elution procedure of Fig. 1 were combined and concentrated as described in the text.

† The protein concentrations were adjusted to about 10 μg/ml before testing on blood agar plates.

‡ These values merely represent the maximal amount of protein injected. The mice did not die from these doses.

Hemolysin activity was tested in the combined samples at a concentration of about 10 μg of protein/ml. Significant activity was found only in sample D, and in the original material before fractionation. Thus, the semiquantitative test indicated that hemolysin activity was not associated with the PF or phospholipase C activities of samples A, B, and C.

Although the results reported above indicate that lethal and hemolysin activities are not associated with either phospholipase, in another step-wise fractionation procedure the lethal and hemolysin factors accompanied PF in ten fractions that were recombined and concentrated after being eluted with 0.4 M tris. However, in agreement with our other tests, no lethality or hemolysin factor was detected in the fractions that contained the phospholipase that inhibited PF activity.

Production of PF inhibitor. It appeared that, under our conditions for growth, shaken cultures produced less PF and phospholipase than those grown statically (Slein and Logan, 1962*a*). Since we found that filtrates prepared from static cultures contained both PF and an inhibitor of PF, it was decided to determine whether shaken cultures contained more of the inhibitor. Filtrates were prepared after the growth of *B. cereus* 6464 and 7004 in media containing phosphate-buffered Casamino Acids or nutrient broth (Difco) plus 0.3% yeast extract, in static or shaken flasks (100 cycle/min). Good growth was obtained in each case. After filtration, the solutions were dialyzed for 23 hr against 0.01 M tris (pH 7.5) at 5 C. The solutions were tested for PF and phospholipase C activities, comparisons being based on volumes rather than protein, since nondialyzable proteins or peptides in the media would interfere (Table 5). With one exception (compare samples 1 and 5), phospholipase activity was higher in static than in shaken cultures. PF activity was higher in static cultures in every case; in fact, no PF was detected in shaken cultures except for relatively low activity in sample 8. It appeared that the phosphate-buffered Casamino Acids medium was better for the production of PF than was the

TABLE 5. *Effect of medium and aeration on the phospholipase, phosphatasemia factor (PF), and PF-inhibitor activities of culture filtrates of Bacillus cereus 7004 and 6464*

Culture	Strain	Sample	Medium	Phospholipase C	PF in vitro	PF inhibition by 0.05 ml
				units/ 0.1 ml	*units/ 0.1 ml*	%
Static	7004	1	Nutrient broth	38	10	NT*
		2	Casamino Acids	134	120	NT
	6464	3	Nutrient broth	59	24	NT
		4	Casamino Acids	57	100	NT
Shaken	7004	5	Nutrient broth	74	0	81
		6	Casamino Acids	60	0	98
	6464	7	Nutrient broth	5	0	22
		8	Casamino Acids	21	5	51†

* Not tested because of the relatively high intrinsic PF activity.

† The over-all inhibition calculated here included the PF activity present in the inhibitory filtrate itself.

nutrient broth-yeast extract medium. However, the results were probably complicated by the presence of varying amounts of PF inhibitor. The filtrates from shaken cultures all contained PF inhibitor. It was not possible to test samples 1 to 4 for inhibitor activity, because of their relatively high PF activities. However, the fractionation with DEAE cellulose, described above, demonstrates that inhibitor may be present even in filtrates from static cultures that have high PF activities. Dialyzed, uninoculated media did not have demonstrable PF inhibitor. Hemolysin activity was found to be greater in filtrates from static than from shaken cultures of either strain of *B. cereus* tested.

It was reported that filtrates of cultures of *B. anthracis* and certain other bacilli grown statically, or *B. cereus* grown statically in the presence of 0.25 M ethanol, did not produce much PF (Slein and Logan, 1962*a*). After the discovery of the PF inhibitor, several of the filtrates that had relatively little PF activity were tested, but no PF-inhibitor was detected. It may be concluded that the strains merely did not produce much PF, and that ethanol inhibited the production of PF by a strain of *B. cereus* that normally produced it in relatively large amounts.

The crude lyophilized lecithinase preparation of Costlow (1958), which had been reported to contain no PF activity (Slein and Logan, 1962*a*), has been found to be a fairly potent PF inhibitor. The protein (15 μg) inhibited PF activity about 70% in the bone-slice test. The absence of PF activity had been ascribed to the loss of PF during 5 years of storage at room temperature. Since the material was prepared from *B. cereus* 7004 that was grown in shaken nutrient broth, it seems probable that it never did contain any demonstrable PF activity.

A larger amount of inhibitor was prepared by growing *B. cereus* 7004 in shaken cultures and concentrating the material by saturation with $(NH_4)_2SO_4$ at 5 C. The precipitate was dissolved, dialyzed, and fractionated on a DEAE cellulose column, with gradient steps of 0.05, 0.2, and 0.4 M tris (pH 8.6). Fractions in each of the three principal absorption peaks (280 mμ) were combined, concentrated, dialyzed, and tested for PF, phospholipase C, and PF-inhibitor activities. None of the three fractions contained PF activity, although experience with preparations from static cultures led us to expect that PF activity should be present in the fractions eluted by 0.4 M tris. All three peaks contained material with inhibitor activity, but most of the inhibitor was present in the nonadsorbed fractions comprising the first peak. Phospholipase C activity was also highest in the nonadsorbed fractions. Thus, it appears that shaken cultures of *B. cereus* may be prepared that have phospholipase C activity devoid of PF activity.

Neither the original material from the shaken cultures of *B. cereus* 7004, nor any of the three fractions tested, had any significant hemolysin activity. No mice were killed by the injection of as much as 75 μg of protein in the $(NH_4)_2SO_4$ precipitate obtained from the shaken cultures.

When the purified inhibitor fraction was mixed with PF in a ratio sufficient to prevent the activity of PF with bone slices, no inhibition of phosphatasemia occurred after intravenous injection of the mixture into a rabbit. This indicates that any complex between the two substances

may be dissociated or destroyed in vivo, or that the concentration of inhibitor may be too low to compete effectively with the PF by the time the materials reach the foci of alkaline phosphatase release from the tissues.

The degree of inhibition of PF activity produced by an amount of inhibitor sufficient to give 50% inhibition in the bone-slice test was not affected by incubation of PF with the inhibitor for various times up to 30 min at 37 C prior to testing. This indicated that the inhibitor was not progressively destroying PF activity, but rather that it was complexing with it or merely competing with it for a substrate in the cell membranes of the bone slices. The competition hypothesis is favored by the results of tests with lecithin, to be described below.

Stability of components of B. cereus culture filtrates. A crude $(NH_4)_2SO_4$ precipitate, dialyzed against 0.02 M phosphate buffer (pH 7.5), and containing about 2.7 mg of protein/ml, was found to have rather stable PF and phospholipase C activities for more than 1 year at about −15 C. PF activity was stable for at least several months at −15 C when the crude preparation was diluted with Ringer phosphate to a protein concentration of 0.1 mg/ml. However, stability was poor when only 4 μg of protein/ml was present during storage. A protamine-treated crude preparation showed no significant loss of PF or phospholipase C activities, during incubation of a solution containing 0.1 mg of protein/ml for 30 min at 37 C, and storage overnight at 5 C. The same stability was found in 0.05 M acetate or tris buffers from pH 5 to 8.5. The crude preparations are stable during lyophilization. Even after storage for more than 5 years at room temperature, the lyophilized inhibitor preparation obtained from R. D. Costlow still had good phospholipase C activity. A purified inhibitor fraction was found to be stable, when incubated at a concentration of 20 μg of protein/ml of 0.005 M tris (pH 7.5) for 30 min at 37 C before testing for PF inhibition and phospholipase C activities. The activities also were not significantly decreased when a solution containing 60 μg of protein/ml of 0.01 M tris (pH 7.5) was frozen and thawed ten times within 3 hr.

Because of the minute amounts of protein required in the bone-slice test for PF activity, it was necessary to determine the stability of PF at high dilutions. A protamine-treated crude material was stable for at least 4 hr at 0 C, at a concentration of 0.5 μg of protein/ml in Ringer phosphate solution. Fraction 65, from the DEAE cellulose column of Fig. 1, was used for studying the stability at a concentration of only 0.04 μg of protein/ml. The preparation was diluted with cold distilled water, Ringer phosphate, 0.01 M tris (pH 7.5), an emulsion of 0.1 mg of soybean lecithin/ml of water, or human serum albumin in water (0.1 mg/ml). The dilute solutions were stored at 0 to 5 C for 1 and 24 hr before testing. Control dilutions were made with Ringer phosphate or albumin, in tris buffer, immediately before samples were added to the flasks with bone slices. PF activity was remarkably stable for 1 hr in any of the diluents, and even after 24 hr only about 50% loss occurred in distilled water. Activity was completely stable for 24 hr when the PF was diluted with albumin as a protective protein, and only about 20% loss occurred during storage in the tris or lecithin solutions. Ringer phosphate appeared to be between water and tris in the stability tests.

Hemolysin and lethal activities were markedly decreased when solutions were heated for 5 min at 100 C. The stability of PF and phospholipase C activities to heat was also tested, in an attempt to determine whether the two might be identical. A relatively crude preparation, containing 0.4 mg of protein/ml, was stable during dialysis for 23 hr against cold distilled water, 0.02 M tris, or phosphate buffer (pH 7.5). After dialysis, samples were heated for 5 min in a boiling-water bath. The material that had been dialyzed against tris or water lost almost all PF and phospholipase C activities. However, the presence of phosphate protected about 20 to 30% of the activities during the heat treatment. These results indicated that phosphate might be combining with the active centers of PF and phospholipase, in the manner by which a natural substrate protects an enzyme from various inactivating agents. The effect of a phospholipase substrate, soybean lecithin, as a protective agent against heat inactivation was tested with purified inhibitor and PF fractions. Samples, containing 18 μg of inhibitor protein or 5 μg of PF protein ± 17 mg of emulsified lecithin/ml of water, were heated for 5 min at 60, 80, or 100 C, chilled in ice, and tested for activities along with unheated control samples. The effect of lecithin on the inhibitor activity could not be tested with

bone slices, since lecithin itself was found to inhibit PF activity (see below). However, the phospholipase C activity of the inhibitor fraction was not significantly protected by lecithin from inactivation at any of the temperatures tested. The amount of inactivation in the absence of lecithin was about the same (25, 55, and 90% at 60, 80, and 100 C, respectively) for either the PF-inhibitor or phospholipase C activity. This indicated that the two activities were identical.

With the purified PF fraction, inactivation of both PF and phospholipase C activities was about 85 to 100% at 80 to 100 C in the absence of lecithin, but this was reduced to about 30 to 65% in the presence of lecithin. It was possible to test PF activity after treatment with lecithin, since the PF activity was so great that the solutions could be diluted sufficiently to avoid inhibition by lecithin during the bone-slice tests.

As with heat inactivation, lecithin was found to inhibit the destruction by trypsin of purified PF and its associated phospholipase C. PF, containing 22 μg of protein/ml of 0.01 M tris (pH 7.5), was incubated for 30 min at 37 C in the presence and absence of crystalline trypsin (about 150 μg/ml), with or without lecithin (9 mg/ml). The solutions were chilled in ice and tested for PF and phospholipase C activities. Both were almost completely destroyed by trypsin, but only 10 to 20% inactivation by trypsin occurred in the presence of lecithin. Trypsin, in the concentrations present in the bone-slice test, did not affect bone phosphatase activity. Lecithin also had no direct effect on PF or phosphatase activities in the concentrations used for the bone-slice test. Thus, lecithin protected both PF and phospholipase C activities in the purified PF fraction in the manner in which a substrate may protect its enzyme from inactivation by heat or trypsin (Trayser and Colowick, 1961). This is evidence that the PF and phospholipase C activities are identical.

Purified inhibitor, unlike PF, was relatively resistant to attack by trypsin, even in the absence of added lecithin. The fact that both PF-inhibition and phospholipase C activities of the inhibitor fraction were resistant to trypsin supports the hypothesis that the activities are identical.

Effect of antiserum on purified fractions. It was reported that antisera, prepared with crude *B. anthracis* lecithinase, inactivated the PF but not the phospholipase of a *B. cereus* culture filtrate (Slein and Logan, 1962*a*). One of the same antisera (AS9) was used in similar tests with purified inhibitor and PF fractions (samples A and C of Table 4). Approximately 31 μg of protein of inhibitor sample A, or 3 μg of PF sample C, were incubated in 0.9% NaCl with 0.15 ml of normal rabbit serum or antiserum, in a volume of 1.05 ml, for 30 min at 37 C. The samples were centrifuged, and the supernatant solutions were tested for inhibitor, PF, and phospholipase activities (Table 6). The PF and phospholipase activities of the PF fraction were markedly decreased by incubation with the antiserum. However, the inhibitor and phospholipase activities of the inhibitor fraction were not significantly reduced by treatment with the antiserum. The approximately 20% greater inhibitor activity found in the presence of antiserum was due to the further inactivation of PF by the 0.007 ml of antiserum added with the inhibitor sample to the bone slice test system. These immunochemical reactions were the basis of the previous results that led to the erroneous conclusion that PF and phospholipase were not identical (Slein and Logan, 1962*a*). The crude culture filtrate probably contained much more phospholipase that did not react with the antiserum than the reactive type associated with PF.

Effect of lecithin on PF and phospholipase C activities. The purified soybean lecithin originally used in our studies was an old preparation ob-

TABLE 6. *Effect of antiserum on the phospholipase, PF-inhibitor, and PF activities of purified fractions of a culture filtrate of Bacillus cereus 6464*

Sample*	Treated with	Activity		
		Phospholipase	PF	PF inhibitor†
C	Normal serum	221	346	—
	Antiserum	34	0	—
A	Normal serum	157	—	99 (340)
	Antiserum	157	—	25 (340)

* The samples were the combined, concentrated, and dialyzed preparations of Table 4. Sample A contained the inhibitor fractions 13 to 20; sample C consisted of the PF fractions 56 to 68.

† The values represent alkaline phosphatase released from bone slices. The values in parentheses were obtained with control samples to which no inhibitor was added.

tained from Nutritional Biochemicals Corp. More recent lots of lecithin contained too much acid-soluble phosphorus for the accurate measurement of phospholipase C activity. Similar lecithin obtained from Mann's Research Laboratories has been satisfactory, so far as the phosphorus content is concerned. However, it was found that the lecithin from Mann's Research Laboratories inhibited the phospholipase activity of a purified PF-inhibitor fraction about 40%, as compared with the older Nutritional Biochemicals Corp. preparation. On the other hand, a purified PF fraction gave the same phospholipase activity with either lecithin. These results are a further demonstration of a difference between the two types of phospholipase C activity, which may be separated on a DEAE cellulose column. The reason for the difference in phospholipase activities obtained with the PF-inhibitor fraction is not known. An inhibitory substance may have been present in the lecithin from Mann's Re-Research Laboratories, or perhaps a less favorable charge distribution existed in the complex of phosphatides present in one lecithin as compared with the other (Dawson and Bangham, 1961).

As was mentioned in the section on stability, lecithin was found to inhibit PF activity in bone-slice tests. The effect of lecithin was not due to any inhibition of bone alkaline phosphatase. It also was not due to the destruction of PF, since activity was restored when a mixture was diluted before testing for PF activity. The effect of lecithin appeared to result from a competition between the emulsified lecithin and a natural substrate in the bone cell membranes for the active site of phospholipase C in the PF fraction. Although the application of solution kinetics is not valid, the effect of lecithin concentration was treated in the manner of a competitive inhibitor and K_i values were calculated from a plot of reciprocal reaction velocities against reciprocal lecithin concentrations, with the molecular weight of lecithin assumed to be 750. From such data, a value of about 1.5×10^{-4} M was obtained as the inhibition constant (K_i) for Mann's lecithin competing with a cell membrane phosphatide for purified PF. Under our experimental conditions, this means that about 0.3 mg of lecithin in 2.5 ml of Ringer phosphate solution with bone slices inhibited the PF activity about 50%. When the phospholipase C activity of the same purified PF fraction was measured with varying concentrations of Mann's lecithin, a K_m value of about 2.7×10^{-2} M was obtained. Thus, about 200 times as much lecithin was required to give half-maximal rate with PF-phospholipase as was required to produce a 50% inhibition of the release of alkaline phosphatase from bone slices by PF. The K_m value calculated for the phospholipase C activity of a purified inhibitor fraction was about 1.3×10^{-2} M with Mann's lecithin.

Effect of PF on bone tissue in vivo. Crude or partially purified PF was very active in producing phosphatasemia, but the presence of a lethal component precluded the use of relatively large amounts of PF for certain in vivo tests. However, with the purified combined fraction C of Table 4 it was possible to obtain a rapid increase of blood alkaline phosphatase to very high levels after intravenous injection into young rabbits. This allowed us to obtain more direct support for the immunochemical data that indicated that bone is a principal source of the phosphatasemic response (Slein and Logan, 1962*a*).

Rabbits were anesthetized with ether, and initial blood samples were taken from marginal ear veins into heparinized tubes. The right hind limb was ligated above the knee, the tibia was amputated and placed in ice. Then, 1 ml of fraction C, containing approximately 30,000 units of PF, was injected into a marginal ear vein. Further blood samples were obtained at intervals, and then the left tibia was removed. Blood was diluted 1:100 with cold distilled water, and 0.1-ml samples were used for the determination of alkaline phosphatase (Slein and Logan, 1962*a*). The tibial epiphyses were sliced, and triplicate samples of each were prepared for the determination of the rate of release of alkaline phosphatase. The results are presented in Table 7. In rabbit 1, the blood alkaline phosphatase increased about tenfold in only 5 min. The release of phosphatase from slices of the tibia, which was removed 5 min after the injection of PF, was significantly higher than that from control slices during the first 15 min in vitro. However, during the second 15 min of incubation, there was little difference between the amounts of phosphatase released. With rabbit 2, the blood phosphatase increased almost 100-fold by 30 min, at which time the second tibia was removed. The release of alkaline phosphatase from bone slices was no longer markedly higher than the control rate at this time; in fact, it was lower than the control

TABLE 7. *Effect of the intravenous injection of 30,000 units of purified PF on rabbit blood and epiphyseal bone alkaline phosphatase*[a]

Rabbit no.	Blood		Bone slices			
	Sample	Alkaline phosphatase	Sample	Phosphatase released spontaneously		Phosphatase released by PF added in vitro[b] (second 15 min)
				First 15 min	Second 15 min	
1	Control	34	Control	351	175	
	Post-PF (5 min)	291	Post-PF (5 min)	564	202	
2	Control	16	Control	192	112	
	Post-PF (15 min)	716				
	Post-PF (30 min)	1,460	Post-PF (30 min)	231	70	
3	Control	48	Control	282	162[c]	1,001[d]
	Post-PF (15 min)	1,318				
	Post-PF (30 min)	2,726	Post-PF (30 min)	277	58[c]	160[d]

[a] Control blood samples and tibiae were removed prior to the injection of PF. The other tibiae were obtained after the last blood samples. Except where noted, triplicate samples of slices from each bone were tested, and the values reported are averages.

[b] Approximately 80 units of a crude PF preparation were added to each flask after the first 15-min samples were taken.

[c] Single samples.

[d] Duplicate samples.

during the second 15-min interval in vitro. Similar results were obtained with rabbit 3. However, in this test, about 80 units of PF were added to two pairs of flasks after the first 15-min samples were taken from the bone-slice incubation. The further release of alkaline phosphatase induced by the PF added in vitro was much greater from slices of the control tibia than from that obtained 30 min after the in vivo administration of PF. These results indicate that alkaline phosphatase is rapidly released from the epiphyseal bone tissue into the bloodstream after the intravenous injection of large amounts of PF. Shortly after injection, the release of phosphatase from the tissue is demonstrably greater than from control slices (rabbit 1), but at a later time the difference is less, or reversed (rabbits 2 and 3). The effect is probably due to the depletion of the alkaline phosphatase content of bone during the in vivo exposure to PF, as is indicated by the lowered response to the addition of PF to slices in vitro (rabbit 3).

Discussion

The presence of at least two enzymes with phospholipase C activity was demonstrated in culture filtrates of *B. cereus* grown under static conditions. The enzymes not only were distinguished by being separable on a DEAE cellulose column, but also by their different sensitivities to trypsin and different degrees of protection by lecithin from heat inactivation. Although they had similar dissociation constants with one preparation of emulsified soybean lecithin as substrate, they had very different activities when tested with two batches of lecithin. Furthermore, the phospholipase that was not readily adsorbed by DEAE cellulose at pH 8.6 was found to have no PF activity and, in fact, was strongly inhibitory to PF in the bone-slice test. The other phospholipase, which was eluted from DEAE cellulose by about 0.4 M tris (pH 8.6), had high PF activity. The different susceptibilities to inactivation by antiserum also distinguished the two phospholipases.

It seems likely that the PF-inhibitor and phospholipase C activities of the nonadsorbed fraction are identical, since neither was very sensitive to inactivation by trypsin, both were inactivated to about the same extent by heat, and both were insensitive to an antiserum that inactivated PF.

Although there was good correlation between the production of PF and phospholipase by several strains of bacilli, it had been concluded that the two activities probably were not identical (Slein and Logan, 1962*a*). However, the present results indicate their identity and explain the apparent discrepancy in our previous publication.

The ratio of the two activities was remarkably constant during elution from DEAE cellulose with a continuous gradient from about 0.4 M to 0.6 M tris (Fig. 1). Both activities were destroyed by trypsin, and the degree of inactivation was reduced similarly for both in the presence of lecithin. A similar protection from heat inactivation by lecithin was found. The inhibition of PF activity by lecithin supports the hypothesis that the release of phosphatase from bone cells is caused by phospholipase activity on the cell membranes. The lecithin appears to compete effectively with a cell membrane phosphatide for the active center of the PF. Further evidence indicating the identity of PF and phospholipase includes the stability of both activities over a wide pH range at 37 C, the slight but significant protection from heat afforded to both activities by dilute phosphate buffer, and a proportional decrease of both activities during storage of a dilute solution of purified PF for several weeks.

The previously reported failure of phospholipase to be significantly inactivated by an antiserum, as compared with PF activity, was probably confused by the presence of more than one phospholipase in the crude culture filtrate that was tested. It is now apparent that the phospholipase associated with PF-inhibitor activity is not susceptible to inactivation by that antiserum, whereas the phospholipase and PF activities in a purified PF fraction are both inactivated by the antiserum.

Concerning the previously assumed differential decay of PF and phospholipase activities during prolonged storage of a lyophilized preparation, it is now evident that the material, which had been prepared from a shaken culture of *B. cereus*, contained only the inhibitor phospholipase activity and probably never had any PF activity.

It is evident that the lethal and hemolysin factors are not related to the phospholipase that inhibits PF activity. Conflicting data have been obtained with regard to the identity of lethality and hemolysin and PF activities. However, since some fractions that had high PF activity appeared to have relatively low lethality and hemolysin activities, it seems likely that the phospholipase associated with PF activity is not identical with the lethality and hemolysin factors. Molnar (1962) separated the toxin of *B. cereus* into two components, neither of which had phospholipase activity when tested by the egg-yolk reaction.

Relatively large doses of purified PF given to rabbits intravenously resulted in the release of alkaline phosphatase from bone epiphyses, so that a significant decrease in the phosphatase content could be demonstrated with bone slices in vitro. This supports the immunochemical evidence for the participation of bone in the development of phosphatasemia. It is probable that other tissues contribute to the phosphatasemic response.

The stabilization of PF activity from heat inactivation by phosphate, or from trypsin inactivation by lecithin, might be useful in the development of further procedures for purification of the enzyme. We plan to investigate the mechanism of action of phospholipase on cell membranes. It was found that PF did not catalyze the release of alkaline phosphatase from cells of *Escherichia coli* that had been induced to form alkaline phosphatase. This indicates that PF does not attack the cell wall of the microorganism sufficiently to allow the escape of the phosphatase that lies between the cell wall and membrane (Malamy and Horecker, 1961).

Altenbern (1962) offered evidence that the production of the toxic complex, including phospholipase, by *B. cereus* is dependent upon the induction of lysogeny, and he suggested that the phospholipase may be essential for the release of phage from inside the bacillus.

Our finding of lower phospholipase activities in shaken cultures of *B. cereus* is probably related to the inactivation of lecithinase by shaking, as recently reported by Kushner (1962).

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