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PHOSPHOLIPASES OF BACILLUS CEREUS: FURTHER CHARACTERIZATION

Milton W. Slein

Gerald F. Logan, Jr.

FEBRUARY 1969

DEPARTMENT OF THE ARMY

Fort Detrick

Frederick, Maryland

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DEPARTMENT OF THE ARMY
Fort Detrick
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TECHNICAL MANUSCRIPT 514

PHOSPHOLIPASES OF *BACILLUS CEREUS*: FURTHER CHARACTERIZATION

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BIOLOGICAL SCIENCES LABORATORIES

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February 1969

ABSTRACT

Results obtained by polyacrylamide gel electrophoresis support the hypothesis that the hydrolyses of phosphatidyl ethanolamine and phosphatidyl choline by the phospholipases C of Bacillus cereus are catalyzed by the same protein, which may be present as isoenzymes. Phosphatidyl inositol and sphingomyelin are hydrolyzed by separate phospholipases that are distinct from the above. Phosphatidyl serine and what appears to be diphosphatidyl inositol are also split by the phospholipases of B. cereus. The yield of phospholipases is much greater in cultures grown statically than in shaken cultures. Zinc and 2-mercaptoethanol had only slight effects on the enzymes specific for phosphatidyl inositol and sphingomyelin, but markedly stimulated phosphatidyl ethanolamine and phosphatidyl choline hydrolysis in a crude preparation of the phospholipases that had been dialyzed against ethylenediaminetetraacetate. A simple microanalytical test has been developed for the detection of phospholipase activity.

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I. INTRODUCTION*

The phospholipase C activity of culture filtrates of Bacillus cereus has often been considered to be a single "lecithinase" with low substrate specificity.[2,3,7,11] We reported the partial purification of at least three phospholipases from both B. cereus and B. anthracis: two that specifically degrade phosphatidyl inositol (PTI) and sphingomyelin (SPH), and one that hydrolyzes both phosphatidyl ethanolamine (PTE) and phosphatidyl choline (PTC).[13] The crude enzyme mixture also splits phosphatidyl serine (PTS) and what appears to be diphosphatidyl inositol, but has no effect on lyso PTC or lyso PTE.[13] Our emphasis on the multiplicity of phospholipases of B. cereus[13,14] has recently been underscored by Pastan, Macchia, and Katzen[10] in the case of Clostridium perfringens. Separation of the PTE and PTC enzymes of B. cereus was not successful, and it was not known whether one or two proteins catalyzed the hydrolyses of these phospholipids.[13,14] Our interest in the phospholipases was mainly concerned with their possible role in the toxicity of culture filtrates of B. anthracis and B. cereus, and in their use in the study of the structure and function of cell membranes.[12-14] However, we have made no attempt at a large-scale production of the enzymes for their purification. The isolation of phospholipases C from B. cereus has been reported by Ottolenghi[9] and by Gollub et al.[4] The present report gives evidence that supports the hypothesis that a single enzyme hydrolyzes both PTE and PTC although isoenzymes may exist. A microanalytical qualitative test has been developed for the detection of phospholipase activity in dilute protein solutions such as those obtained during fractionation on N,N'-diethylaminoethyl (DEAE) cellulose or gel electrophoresis columns. Further properties of the phospholipases of B. cereus are reported.

II. MATERIALS AND METHODS

A. QUANTITATIVE PHOSPHOLIPASE ASSAY

The crude phospholipases of B. cereus strain 6464 and fractions partially purified on DEAE cellulose columns were prepared from the supernatant fluid of cultures grown statically as previously described.[12,13] The general chromatographic procedure for the quantitative assay of specific phospholipases has also been reported.[13,14] Most tests were made with a buffered emulsion of "purified" soybean lecithin, which contained PTE, PTC, lyso PTC, PTI, and diPTI, and was supplemented with SPH.[13-14] The reaction was usually carried out in a final volume of 0.10 or 0.12 ml containing approximately 0.3 mg each of PTE, PTC, and SPH (estimated chromatographically by relative acid fuchsin color intensities as compared with known amounts

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of SPH), and 0.1 mg of PTI (by analysis[13]). PTS was an impure bovine brain product obtained from Sigma Chemical Co. (Folch fraction III, lot 113B-1220). Less than 1.6 mg of PTS was included in the reaction mixture since several other unidentified phospholipids were present as contaminants in the emulsion. Except where noted, activities were measured in the presence of about 0.0004 M $ZnCl_2$ and 0.001 M 2-mercaptoethanol, the enzyme being added to the solution of zinc and thiol at room temperature 1 to 20 minutes <u>before</u> adding 0.05 ml of buffered substrate emulsion and placing the reaction mixture in a water bath at 37 C. Metal ions and reducing agent were not included in the specific phospholipase determinations previously reported by us.[13,14] For tests with metal ions, a dialyzed, concentrated, crude preparation of the phospholipases was further dialyzed against cold 0.001 M ethylenediaminetetraacetate (EDTA), 0.005 M phosphate, pH 7.2, for 15 hours and then against two changes of cold 0.005 M phosphate for 8 hours in order to remove most of the EDTA and any metal ions bound to it.

The specific activities (per cent hydrolyzed per μg protein per 10 minutes) merely indicate the relative activities of the different phospholipases under the conditions of assay and are not absolute values. This results partly from the fact that the concentrations of the various phospholipids in the mixture are not known in terms of molarity. Furthermore, interactions of the individual enzymes with the various substrates and unknown contaminating substances probably affect the specific activities.

B. MICROANALYTICAL TEST FOR PHOSPHOLIPASE ACTIVITY

A spot test was developed for the qualitative detection of phospholipase C activity. Spreading of droplets was prevented by applying four circles of Dow Corning silicone grease to a microscope slide with the flat end of a piece of glass tubing (6 mm I.D.). In each circle was placed 0.01 ml of an emulsion of phospholipids in 0.2 M phosphate, pH 7.2.[13,14] To each sample was added 0.01 ml of a dilute solution of phospholipase and the mixture was stirred with a fine-tipped glass rod. A thick layer of grease was spread around the edges of a second slide, which was gently pressed down over the first slide until the surface of the upper slide contacted the droplets. The slides were incubated at room temperature (about 25 C) for several minutes or hours in order to detect phospholipase activity as indicated by an increase in turbidity of the emulsion (Fig. 1). In this manner, many dilute samples from a fractionation procedure may be rapidly surveyed for phospholipase. In a typical example, 0.2 μg protein gave detectable activity in 20 to 30 minutes.

It is also possible to detect phospholipase C activity by the development of turbidity after placing droplets of enzyme solution on the surface of a thin layer of a buffered emulsion of phospholipids in 1% Special Agar-Noble (Difco). The gelled emulsion may be prepared on microscope slides and stored

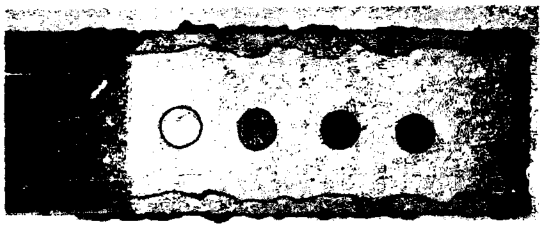

FIGURE 1. Microtest for Phospholipase Activity. Each droplet contained an emulsion of buffered phospholipids plus, from left to right: water, 0.75 μg, 1.0 μg, and 2.5 μg of crude phospholipase proteins of B. cereus. The slide was incubated for 2 hours at about 24 C.

in a moist chamber. Such an emulsion in agar may also be used for locating bands of phospholipase in fresh, unstained, analytical polyacrylamide gels after electrophoretic separation of the proteins. However, diffusion of enzyme in the moist interface between the gel and agar surfaces may obscure the results.

C. GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed at room temperature with a Canalco* model 6 apparatus for analytical purposes, and at about 5 C in a Prep-Disc* apparatus for preparative fractionations. Both procedures were carried out with only a separating gel, usually containing 4% acrylamide with ammonium persulfate as gelling catalyst. Sample solutions containing protein in 5% sucrose and 0.01 M phosphate, pH 7.2, were layered on the gel surfaces under the upper electrolyte at the cathode. Electrolyte used in gel preparation, electrode chambers, and for eluting fractions from the Prep-Disc apparatus was adjusted to give a final concentration of 0.025 M tris(hydroxymethyl)aminomethane (Tris) and 0.19 M glycine (about pH 8.6 at 22 C, pH 8.8 at 10 C). Prep-Disc electrophoresis was carried out in the PD-2/150 apparatus with a gel column 3 to 6 cm long and a current-stabilized power supply at a maximum of 15 to 20 milliamperes. Fractions of 1 to 5 ml were collected at about 1 ml per minute in a model 3R-40002 refrigerated *fraction collector.*** Collection of fractions was begun when the bromophenol blue marker dye disc was about to elute from the gel. Fractions were dialyzed in the cold against 0.005 M phosphate, pH 7.2, and were stored frozen. Analytical gels were approximately 6 cm long and 0.5 cm in diameter, and a maximal current of 2 milliamperes per gel was used. Protein discs were stained with 0.1% Amido Black 10B in 7% acetic acid. Excess background stain was removed by soaking the gels in 7% acetic acid. It was possible to elute samples from unstained gel slices by crushing the gel in a Ten Broeck tissue grinder with 0.005 M phosphate, pH 7.2, allowing the protein to diffuse out of the particles, and removing the gel by centrifugation.

D. OTHER PROCEDURES

The method of Waddell[15] was used for estimating the distribution of protein in the dilute concentrations present in fractions. More accurate protein determinations were made by the procedure of Lowry et al.[8] When desired, fractions were combined, transferred to cellophane dialysis tubing (10-mm flat width), concentrated by pervaporation at room temperature in an air stream from an electric fan, and dialyzed against cold 0.005 M phosphate, pH 7.2.

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* Canal Industrial Corp., Rockville, Md.

** Buchler Instruments, Inc., Fort Lee, N. J.

III. RESULTS

A. FRACTIONATION BY ELECTROPHORESIS IN POLYACRYLAMIDE GEL

Many attempts have been made to resolve the protein that catalyzes the hydrolyses of PTE and PTC. The unique resolving power of polyacrylamide gel electrophoresis has been used with the concentrated crude phospholipases and with material partially purified by gel electrophoresis or by chromatography on DEAE cellulose.[12,13] Two examples of the type of results obtained are presented. Figure 2 gives the profile of protein distribution obtained by fractionation of 5 mg of the crude phospholipases. Approximately 40% of the protein was recovered in the 20 fractions (100 ml). The profile, obtained by direct spectrophotometry of the dialyzed fractions,[15] does not give the detail found by analytical gel electrophoresis of the fractions after they were selectively combined and concentrated (Fig. 3). The data in Table 1 show that very little phospholipase was present in fractions 1 to 3, which contained the most protein (Fig. 2). PTI activity was highest in fractions 5 to 7, while SPH activity was best in fractions 6 and 7. PTI phospholipase was partially separated from that of SPH in fractions 4 and 5. PTE and PTC phospholipase was concentrated in fractions 8 and 11, indicating the existence of two isoenzymes. The fact that the ratios of PTE:PTC activities were relatively constant in fractions 4 to 12 supports the idea that the same protein catalyzes the hydrolyses of both phospholipids. The distribution of proteins (Fig. 3) is such that, if a specific phospholipase were required for the hydrolysis of each phospholipid, the ratios PTE:PTC would be much more variable. The relatively low activities for PTE and PTC in the fractions as compared with those in the crude material are rather disappointing, but are probably the result of partial inactivation of the enzymes during electrophoresis. In separate tests with PTS, activity was associated with the fractions that also had PTE and PTC activities.

Material having the best PTE and PTC activities from several Prep-Disc and DEAE cellulose fractionations was combined. The preparation had no PTI activity, but was contaminated with SPH phospholipase. About 0.65 mg of the protein was refractionated by gel electrophoresis and the profile of the eluted material is given in Fig. 4. Approximately 50% of the protein was recovered in the 56 fractions (112 ml). The first sharp peak (fractions 13 to 19) corresponds to the main peak of Figure 2 and had relatively low phospholipase activity (Table 2). The broad, heterogeneous portion comprising the remaining 37 fractions was divided into four regions for further analysis as is shown in Table 2. PTE and PTC activities were concentrated in fractions III to VI, while SPH was best in fraction II. Here, again, no significant variation in the ratios of PTE:PTC activities occurred during fractionation although the material was enriched with these two phospholipase activities before fractionation. Fractions III to VI were combined, concentrated, and redialyzed. Data obtained with these combined fractions show the effect of SPH on PTE and PTC activities; approximately 25 to 30% inhibition was produced by SPH without any marked

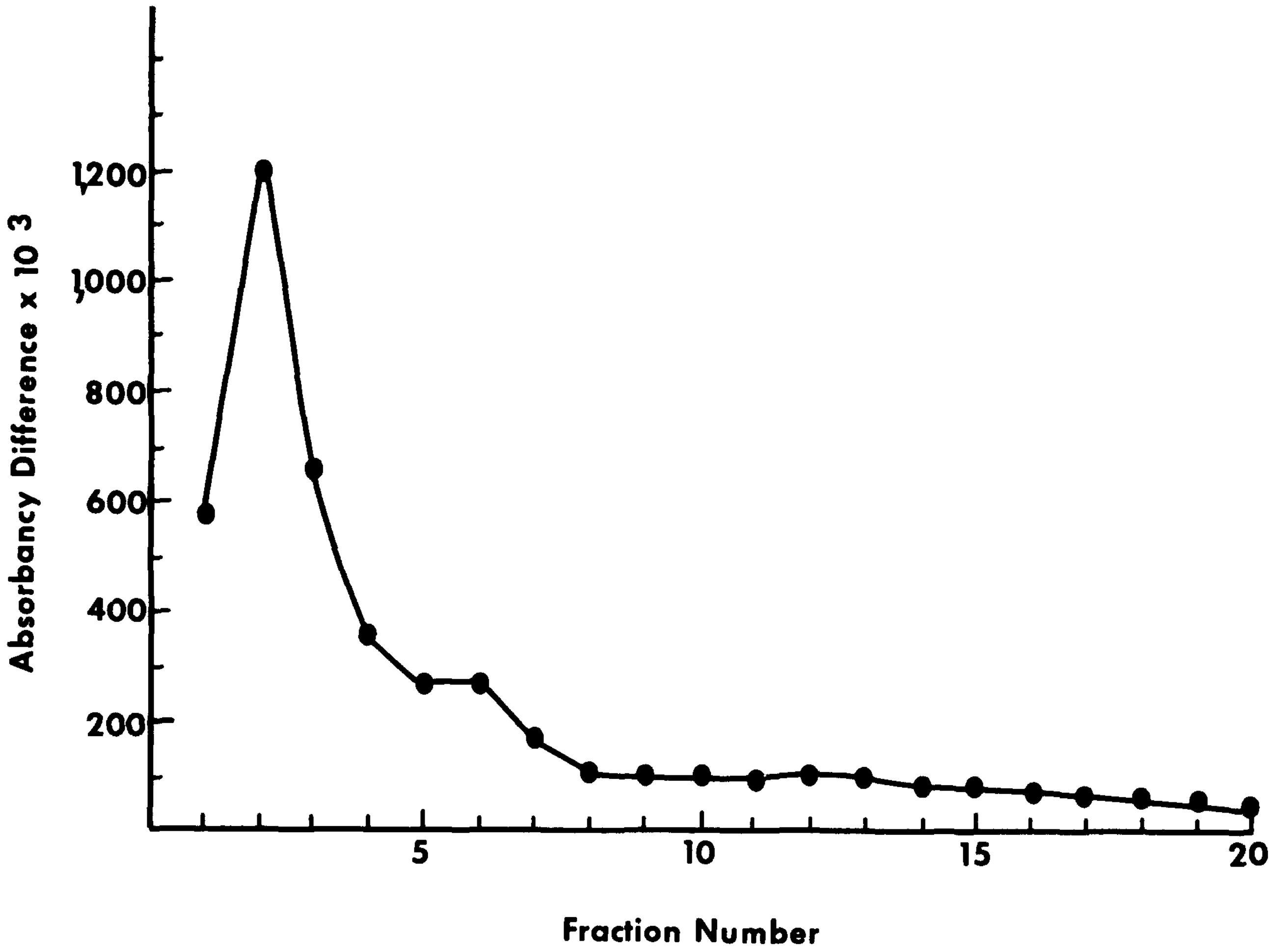

FIGURE 2. Profile of Protein Eluted During Fractionation of Crude Phospholipases of B. cereus by Polyacrylamide Gel Electrophoresis. Five-ml fractions were collected, dialyzed, and the differences in absorbancy were calculated from readings at 215 and 225 nm.

FIGURE 3. Stained Protein Discs after Analytical Polyacrylamide Gel Electrophoresis of the Phospholipases of B. cereus. The cathode was above the gels as aligned here. The four gels on the left contained 10 to 40 μg of protein in fractions I, II, III to VI, and the original partially purified material, respectively, of Table 2. Proteins (0.1 mg) of a crude phospholipase preparation are shown in the fifth gel from the left. Sixteen discs were visible in this gel, but several of the paler ones were not reproduced during photography. The seven gels to the right of the crude material represent 15 to 36 μg protein of fractions 1, 2, 3, 4, 5, 6, and 11, respectively, of Table 1.

TABLE 1. SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED BY GEL ELECTROPHORESIS OF A CRUDE PREPARATION OF PHOSPHOLIPASES FROM B. CEREUS

Fraction Number	Numbers of Original Fractions Combined[a]	Specific Activity[b]				PTE/PTC
		PTI	PTE	PTC	SPH	
1	-	4.5	0	0	0	-
2	-	1.4	0	0	0	-
3	-	3.0	0	0	0.8	-
4	-	19.0	1.5	3.3	2.8	0.5
5	-	34.0	2.4	3.3	6.9	0.7
6	-	33.3	2.9	4.9	12.4	0.6
7	7 - 8	29.0	5.8	8.1	12.5	0.7
8	9 - 10	3.2	7.7	10.0	3.8	0.8
9	11 - 12	-	6.1	8.6	1.3	0.7
10	13 - 14	-	6.1	8.6	1.1	0.7
11	15 - 17	-	9.0	11.7	1.7	0.8
12	18 - 20	-	5.2	8.1	0	0.6
Crude	-	13.9	12.1	15.5	4.2	0.8

a. Five-ml fractions (Fig. 2) were combined, concentrated and redialyzed. Fractions 1 to 6 were only concentrated and redialyzed.

b. Per cent hydrolyzed per μg protein per 10 minutes. PTI activity was not measured in fractions 9 to 12.

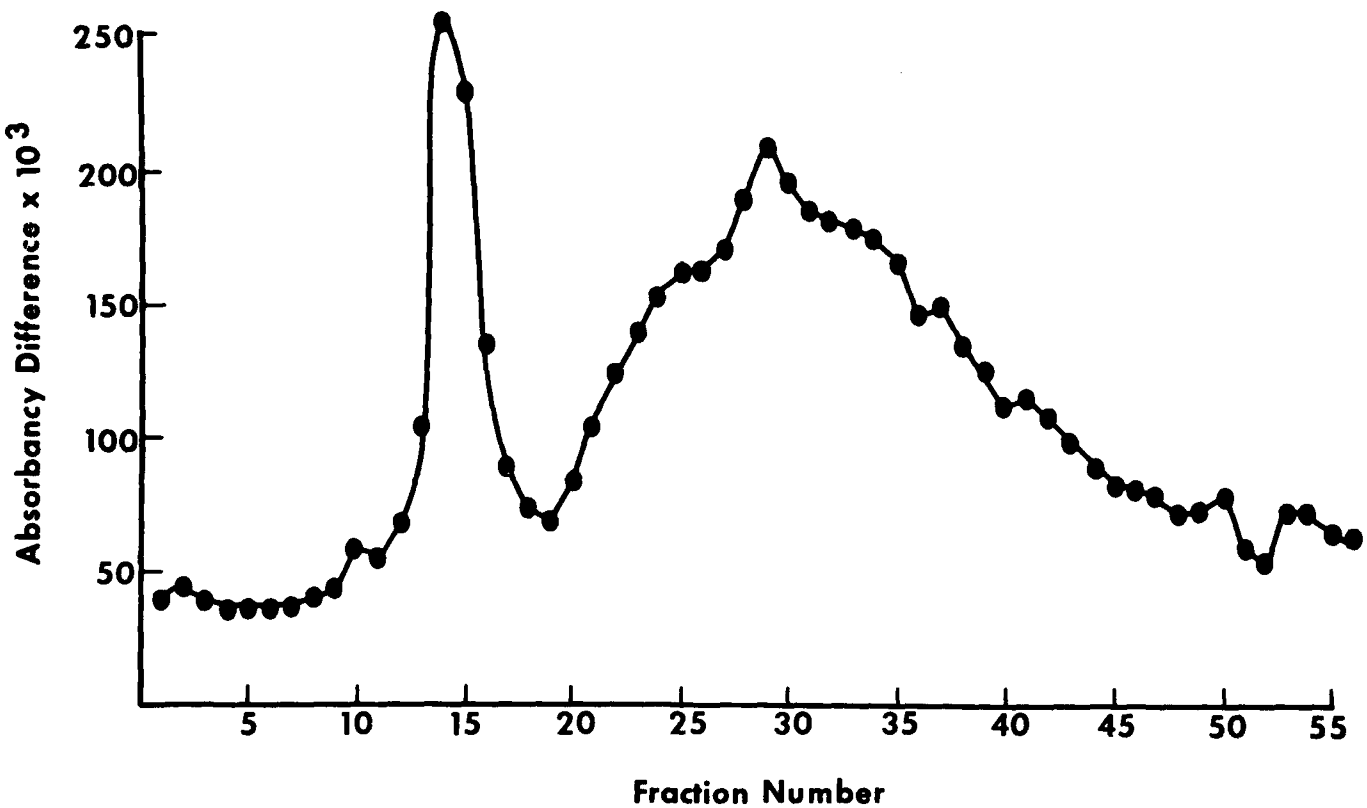

FIGURE 4. Profile of Protein Eluted During Fractionation of Partially Purified Phospholipases of B. cereus by Polyacrylamide Gel Electrophoresis. Two-ml fractions were collected and treated as in Figure 2.

TABLE 2. SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED BY GEL ELECTROPHORESIS AFTER COMBINING PARTIALLY PURIFIED PHOSPHOLIPASES OF B. CEREUS HAVING MOSTLY PTE AND PTC ACTIVITIES

Fraction Number	Numbers of Original Fractions Combined[a]	Addition[b]	Specific Activity[c]				PTE/PTC
			PTI	PTE	PTC	SPH	
I	1 - 12	SPH	0	1.5	1.9	2.0	0.8
II	13 - 19	SPH	0	4.1	5.3	5.5	0.8
III	20 - 26	None	N.M.[d]	21.0	25.1	N.M.	0.8
IV	27 - 35	None	N.M.	22.5	27.4	N.M.	0.8
V	36 - 46	None	N.M.	31.5	37.6	N.M.	0.8
VI	47 - 56	None	N.M.	21.9	25.1	N.M.	0.9
III - VI	20 - 56	None	N.M.	17.1	20.4	N.M.	0.8
		SPH	0	11.4	15.5	1.6	0.7
Before fractionation	-	SPH	0	36.9	42.2	5.0	0.9

a. Two-ml fractions (Fig. 3) were combined, concentrated, and redialyzed.
b. SPH was included in the emulsion of phospholipid substrates where indicated.
c. Per cent hydrolyzed per μg protein per 10 min.
d. N.M. = not measured.

change in the ratio of PTE:PTC. Some loss of PTE and PTC activities occurred when fractions III to VI were combined as shown by the lower values obtained in the absence of SPH as compared with values for the individual fractions III to VI that were also measured in the absence of SPH. In another test it was determined that SPH had no marked effect on the hydrolysis of PTI by a fraction containing PTI phospholipase.

The resolving power of polyacrylamide gel electrophoresis is demonstrated in Figure 3. The protein discs were stained after analytical gel electrophoresis of several fractions that had been obtained by preparative gel electrophoresis for the data in Tables 1 and 2. Fifteen to 20 protein discs are usually seen in the pattern obtained with about 0.1 mg of the crude phospholipase protein. The darkest disc remains at the cathode end of gels containing 4% or more acrylamide, but the protein of this disc readily moves toward the anode in a 3% gel. However, 3% gels are more easily damaged and are much more difficult to handle than those prepared with 4% acrylamide. Somewhat greater resolution of proteins occurs in 7 or 8% gels, but decreased mobility prolongs the time required for a preparative fractionation. The progress of fractionation is demonstrated by the series of gels representing most of the fractions of Table 1. It is not possible to assign specific phospholipase activities to any of the discs with certainty until single proteins have been isolated that give sharp discs in several gels ranging from 4% to 10% acrylamide. Fraction 6 contains a heavy protein disc that might be associated with PTI or SPH phospholipase (Table 1), but the corresponding disc is present in fraction II, which was prepared from material that had essentially no PTI activity (Table 2). Thus, it is possible that the dark disc in fraction 6 represents SPH phospholipase, and that one of the paler discs in fraction 5 is the PTI enzyme. PTE and PTC phospholipase activities have been consistently related to the discs shown in fractions 8, 11, and III to VI, but no definite assignment can be made at present.

B. PRODUCTION OF PHOSPHOLIPASES IN STATIC AND SHAKEN CULTURES

It was previously reported that phospholipase C was generally obtained in greater yield from two strains of _B. cereus_ when they were grown in static rather than in shaken cultures.[12] We have always grown _B. cereus_ in static cultures of phosphate-buffered Casamino Acids for the production of phospholipases. Recently, we studied the effect of shaking cultures on the production of the various specific phospholipases, since the earlier report was based on the nonspecific release of acid-soluble phosphorus from a mixture of phospholipids as a measure of phospholipase activity. The results obtained with _B. cereus_ strain 6464 are summarized in Table 3. It is obvious that all five phospholipase activities were highest when the protein was isolated from cultures grown statically in Casamino Acids. Furthermore, cultures grown statically in nutrient broth with yeast extract also produced significantly more phospholipases than did shaken cultures.

TABLE 3. SPECIFIC ACTIVITIES OF PHOSPHOLIPASES C IN SUPERNATANT FLUIDS FROM STATIC AND SHAKEN CULTURES OF B. CEREUS

Culture[a]	Specific Activity[b]				
	PTI	PTE	PTC	SPH	PTS
Nutrient broth, static	1.7	1.7	2.1	0.2	0.15
Nutrient broth, shaken	0.04	0.4	0.5	0.01	0.04
Casamino Acids, static	13.4	11.0	15.0	1.8	0.65
Casamino Acids, shaken	0.02	0.36	0.5	0.03	0.08

a. Cultures were grown in 100 ml of medium in 1-liter Erlenmeyer flasks for 24 hours at 37 C after being inoculated with about 2×10^8 spores.
b. Per cent hydrolyzed per µg protein per 10 minutes.

C. EFFECT OF METAL IONS AND 2-MERCAPTOETHANOL ON PHOSPHOLIPASE C ACTIVITIES

The addition of Zn^{+2} to crude phospholipases that had been repeatedly dialyzed against dilute Tris or phosphate buffer resulted in only a moderate stimulation of activities. However, when a crude preparation of the phospholipases was dialyzed against EDTA as described in Section II, a marked stimulation of PTE and PTC activities by Zn^{+2} was obtained, although added metal ion was not required for activity. The effects of 5×10^{-4} M divalent cations on the phospholipase activities of a crude preparation that had been dialyzed against EDTA are shown in Table 4. No mercaptoethanol was present in these tests. None of the cations tested had any appreciable effect on the phospholipases that hydrolyze PTI or SPH with the exception of the 51% inhibition of PTI activity by Co^{+2} and the 33% inhibition of SPH activity by Mg^{+2}. However, PTE and PTC activities were significantly inhibited by all of the cations except Ca^{+2} and Zn^{+2}. Zn^{+2} was much more effective in stimulating the PTE and PTC phospholipase activities than was Ca^{+2}. The effects of various concentrations of Zn^{+2} on the EDTA-treated phospholipases in the absence of thiol are given in Table 5. PTI and SPH phospholipases were not markedly affected, whereas the PTE and PTC activities were at least doubled by 10^{-5} M Zn^{+2}. PTE and PTC activities were maximal at about 10^{-4} M Zn^{+2}, which produced a threefold to fourfold increase in activities over those obtained in the absence of added Zn^{+2}.

TABLE 4. EFFECT OF DIVALENT METAL IONS ON THE ACTIVITIES OF AN EDTA-TREATED PREPARATION OF CRUDE PHOSPHOLIPASES OF B. CEREUS[a]

Metal Ion, 5×10^4 M	Stimulation or Inhibition, %[b]			
	PTI	PTE	PTC	SPH
Ca	+8	+22	+29	-6
Co	-51	-44	-64	+10
Mg	-5	-22	-39	-33
Mn	-12	-100	-71	-13
Zn	-11	+199	+77	-13

a. The phospholipases (4.3 μg protein) were treated with the metal chlorides at about 25 C for 1 minute before adding the substrate emulsion and incubating at 37 C for 25 minutes. The metal ion concentration was that present in the complete reaction mixture. No thiol was added in these tests.

b. The values indicate the effects produced compared with control samples to which no metal ion was added.

TABLE 5. EFFECT OF Zn^{+2} CONCENTRATION ON THE ACTIVITIES OF AN EDTA-TREATED PREPARATION OF CRUDE PHOSPHOLIPASES OF B. CEREUS[a]

Final Molarity of $ZnCl_2$	Stimulation or Inhibition, %[b]			
	PTI	PTE	PTC	SPH
10^{-6}	0	+20	-6	-13
10^{-5}	-6	+100	+147	-5
10^{-4}	-22	+390	+323	+11
10^{-3}	-26	+370	+300	-11

a. Procedure as for Table 4 except that incubation was 15 minutes for PTI and 30 minutes for the other phospholipids.

b. The values indicate the effects produced compared with control samples to which no $ZnCl_2$ was added.

The effects of Zn^{+2} and the reducing agent, 2-mercaptoethanol, are presented in Table 6. Here again, the crude phospholipases were treated with EDTA prior to testing. PTS hydrolysis was measured after incubation for 90 minutes with 129 μg of enzyme protein, whereas the other activities were measured after only 25 minutes with 4.3 μg of protein. The relatively weak activity with PTS may have resulted from the presence of contaminating substances in the emulsion. No PTS was hydrolyzed in the absence of added Zn^{+2}, and the addition of 2-mercaptoethanol did not further increase the activity obtained with Zn^{+2} alone. In contrast, the other phospholipases were partially active in the absence of these additions. Zn^{+2} or thiol or both produced about equal, moderate stimulations of PTI phospholipase of 30 to 35%, although Zn^{+2} had produced slight inhibitions in the tests characterized in Tables 4 and 5. SPH phospholipase was slightly inhibited by Zn^{+2}, and the moderate stimulation by the thiol also seemed to be slightly inhibited by Zn^{+2}. On the other hand, PTE and PTC phospholipase activity was tripled by either Zn^{+2} or mercaptoethanol and was stimulated slightly further in the presence of both.

There did not seem to be an appreciable difference in the effects produced by Zn^{+2} and thiol when the enzymes were incubated with them for 1 or 20 minutes at room temperature before adding the substrates for reaction at 37 C. However, when the substrate emulsion was added to the Zn^{+2} and thiol 2 to 15 minutes before adding the enzyme, a 25 to 65% inhibition of PTE and PTC phospholipase activity was produced.

In contrast to the results obtained above with crude phospholipase preparations, a partially purified fraction containing PTE and PTC phospholipase had no significant activities in the absence of added Zn^{+2} or thiol even though the fraction had only been dialyzed against 0.005 M phosphate, pH 7.2, in the absence of EDTA.

IV. DISCUSSION

Several lines of evidence support the hypothesis that the hydrolyses of PTE and PTC by the phospholipases of *B. cereus* are catalyzed by a single enzyme. Previously reported results[13] bearing on this include the failure to separate the activities by chromatography on columns of DEAE cellulose and the resistance of both activities to trypsin. Further support is given by the fact that the ratios of PTE:PTC activities are relatively constant over the range of fractions obtained by gel electrophoresis of the crude mixture of phospholipases or of partially purified material. The similar responses of PTE and PTC activities to divalent cations and 2-mercaptoethanol are distinct from those of the PTI and SPH activities. Furthermore, both PTE and PTC activities are inhibited to about the same extent in the presence of SPH, while PTI activity is not significantly affected by SPH. Although the same protein appears to hydrolyze both PTE and PTC, gel electrophoresis has given some evidence for the existence of isoenzymes in the mixture of

TABLE 6. EFFECT OF Zn^{+2} AND 2-MERCAPTOETHANOL ON THE HYDROLYSIS OF PHOSPHOLIPIDS BY AN EDTA-TREATED PREPARATION OF THE CRUDE PHOSPHOLIPASES OF *B. CEREUS*[a/]

Additions	Phospholipid Hydrolyzed, %				
	PTS	PTI	PTE	PTC	SPH
None	0	66	18	26	29
$ZnCl_2$	30	85	55	78	24
2-mercaptoethanol	0	89	55	75	42
Both	27	89	71	85	36

a. The phospholipases were treated with $ZnCl_2$ or thiol or both for 1 minute at about 25 C before adding the substrate emulsion and incubating at 37 C. With PTS, 129 μg protein were incubated for 90 minutes; with the mixture of other phospholipids, only 4.3 μg protein were incubated for 25 minutes. The final concentrations of the additions were about 6 x 10^{-4} M thiol and 2 x 10^{-4} M $ZnCl_2$.

crude phospholipases. Gel electrophoresis has limited the number of protein bands possibly associated with PTE and PTC phospholipase, but we have not yet identified any specific protein disc with the activities. The existence of two other phospholipases that specifically hydrolyze PTI and SPH has been demonstrated by their separation on DEAE cellulose[13] and by gel electrophoresis (Tables 1 and 2). They also respond differently to divalent cations, especially Co^{+2} and Mg^{+2}. The phospholipases of *B. cereus* strain 6464 also hydrolyze PTS and diPTI, but apparently do not attack lyso PTC, lyso PTE, or cardiolipin. It is possible that other phospholipids that have not been tested are hydrolyzed by the *B. cereus* enzymes.

Tests reported here and in a previous paper[12] show that the yield of phospholipases from *B. cereus* is much better in the supernatant fluid from static than from shaken cultures. This is probably related to the inactivation of "lecithinase" by shaking as reported by Kushner.[6] Johnson and Bonventre[5] reported that the optimal amount of phospholipase C was obtained within a rather limited time of incubation of *B. cereus* in shaken cultures.

Ottolenghi[9] reported that phospholipase C from B. cereus is an enzyme that requires zinc and thiol groups for full activity. His results were obtained with assays using egg yolk or purified lecithin as substrates. Zinc was the most effective cation for reactivating the EDTA-treated enzyme. Although glutathione and cysteine could not replace zinc in reactivating the EDTA-treated enzyme, they restored about 60% of the activity lost by prolonged dialysis against distilled water. The further addition of Zn^{+2} restored only slightly more of the latter activity. If our results with PTC (lecithin) are compared with those of Ottolenghi, marked stimulation of the EDTA-treated enzyme was produced by either Zn^{+2} or 2-mercaptoethanol, and only slightly more by both. It is possible that our preparations had been partially oxidized during several dialyses against dilute Tris or phosphate buffers so that they responded to both the reducing agent and Zn^{+2} after treatment with EDTA. The rather slight inhibition of the phospholipase C of B. cereus reported by Johnson and Bonventre may be related to their having treated the substrate rather than the enzyme with chelating agents.[5] However, they did find complete inhibition of the phospholipase of C. perfringens by chelating agents under the same conditions of treatment.

Pastan, Macchia, and Katzen have recently reported[10] the isolation from C. perfringens cultures of a phospholipase specific for SPH. The purified enzyme appears to be different from the corresponding phospholipase in our crude preparation from B. cereus in that it is inhibited more strongly by 10^{-3} M $ZnCl_2$ (80% compared with about 10%). However, it is not possible to unequivocally compare the two enzymes unless it can be done under identical conditions of preparation and assay. Another phospholipase C has been extracted in impure form from an alga.[1] This enzyme appears to be quite different from those obtained from bacilli or C. perfringens in being heat-labile, being unaffected by EDTA, and having a lower pH optimum.

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13. ABSTRACT

Results obtained by polyacrylamide gel electrophoresis support the hypothesis that the hydrolyses of phosphatidyl ethanolamine and phosphatidyl choline by the phospholipases C of Bacillus cereus are catalyzed by the same protein, which may be present as isoenzymes. Phosphatidyl inositol and sphingomyelin are hydrolyzed by separate phospholipases that are distinct from the above. Phosphatidyl serine and what appears to be diphosphatidyl inositol are also split by the phospholipases of B. cereus. The yield of phospholipases is much greater in cultures grown statically than in shaken cultures. Zinc and 2-mercaptoethanol had only slight effects on the enzymes specific for phosphatidyl inositol and sphingomyelin, but markedly stimulated phosphatidyl ethanolamine and phosphatidyl choline hydrolysis in a crude preparation of the phospholipases that had been dialyzed against ethylenediaminetetraacetate. A simple microanalytical test has been developed for the detection of phospholipase activity.

14. Key Words

*Bacillus cereus
*Phospholipase
*Electrophoresis
Catalysis
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