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Characterization of the Phospholipases of *Bacillus cereus* and Their Effects on Erythrocytes, Bone, and Kidney Cells

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ABSTRACT

SLEIN, MILTON W. (Fort Detrick, Frederick, Md.), AND GERALD F. LOGAN, JR. Characterization of the phospholipases of *Bacillus cereus* and their effects on erythrocytes, bone, and kidney cells. J. Bacteriol. **90**:69-81. 1965.—Culture filtrates of *Bacillus cereus* contain phospholipases that split phosphoryl choline, phosphoryl ethanolamine, and phosphoryl inositol from the phospholipids phosphatidyl choline (PTC), sphingomyelin, phosphatidyl ethanolamine (PTE), and phosphatidyl inositol (PTI). It is possible that one enzyme catalyzes the degradation of PTE and PTC, but the other phospholipases appear to be separate entities. Some activity on phosphatidyl serine has also been noted. Quantitative paper chromatography has been used for characterizing the phospholipases that are separated on *N,N'*-diethylaminoethyl cellulose columns. A procedure for the analysis of inositol is included. A sensitive kidney cortex homogenate test is described that depends on the release of alkaline phosphatase for the measurement of phosphatasemia factor (PF) activity associated with the phospholipases. The effects of the phospholipases on erythrocytes, kidney, and bone cells are discussed. Hemolysin activity is inhibited by crude soybean "lecithin," but hemolysis does not seem to be identical with PTE- or PTC-phospholipase activity. PF activity is also inhibited by the "lecithin." Highest PF activity is associated with PTI-phospholipase. The phospholipase fractions differ in their sensitivities to trypsin. Phospholipases with similar properties have been obtained from culture filtrates of *B. anthracis*.

The partial purification of two phospholipases from culture filtrates of *Bacillus cereus* has been reported (Slein and Logan, 1963). One of them resulted in a marked phosphatasemia after intravenous injection into rabbits; bone appeared to be a major source of the excess serum alkaline phosphatase (Slein and Logan, 1962, 1963). The second phospholipase inhibited the release of phosphatase from epiphyseal bone slices by the first (Slein and Logan, 1963). Results in the present paper indicate that other phospholipases may be isolated from culture filtrates of *B. cereus* and that each of them probably has specific phospholipid substrate requirements. The relationship of the phospholipases to hemolysis and to the release of alkaline phosphatase from bone and kidney cortex cells has been investigated.

MATERIALS AND METHODS

Strain 6464 of *B. cereus* was originally obtained from the American Type Culture Collection. General procedures for the preparation of culture filtrates and for the isolation of phospholipases have been described (Slein and Logan, 1963). The method for measuring phosphatasemia factor (PF) activity with bone slices in vitro was included in the same report.

Homogenate tests for PF activity. The bone slice test is about 100 times as sensitive as the phospholipase method that depends on the liberation of acid-soluble phosphorus from lecithin. The bone slice test for PF activity is also more specific, since it is based on the degradation of phospholipids in the cell membrane for the release of alkaline phosphatase. A phospholipase that splits acid-soluble phosphorus from lecithin may not necessarily have PF activity (Table 2 in Slein and Logan, 1963). However, the preparation of bone slices is time-consuming, and the number of samples that can be tested is rather limited. It was found that homogenates of rabbit bone epiphyses or kidney cortex could be used for the measurement of PF activity. The kidney cortex was chosen for general use, but some comparisons were made with bone homogenates that gave results analogous to those obtained with bone slices. Rabbits weighing about 1 kg were anesthetized with chloroform and were exsanguinated. The kidneys were chilled in ice, split lengthwise, demedullated, and the cortex was stored in ice-cold

Ringer phosphate solution (*p*H 7.5). The cortex was blotted, weighed, and an amount of up to 2.5 g was homogenized with 25 ml of cold Ringer phosphate solution in a VirTis 45 homogenizer for 0.5 min with the rheostat set on 50 (a speed of about 16,000 rev/min when measured with the blades out of solution). Higher speeds or longer times resulted in too much cell damage and loss of phosphatase. The homogenate was centrifuged for 5 min at about 200 × *g* at 5 C, and the particulate fraction was washed twice with cold Ringer phosphate solution. The precipitate was suspended with 5 ml of cold Ringer phosphate solution for each gram of cortex that had been homogenized. Samples (0.5 ml) were pipetted into 1 × 10-cm tubes in an ice bath and 0.2 ml of Ringer phosphate or other solutions to be tested was added to each. The tubes were stoppered, placed at a 45° angle in a rack in a Dubnoff Metabolic Shaker, and were shaken at about 160 cycles/min for 30 min at 37 C. The samples were chilled in ice, centrifuged at 5 C for 6 min at about 2,000 × *g*, and 0.1-ml portions were diluted with cold distilled water (usually 1:5 to 1:10). To prevent the inclusion of any floating particles, bits of cotton wool were wound onto the tapered, ground tips of pipettes used for sampling the supernatant solutions. Duplicate 0.1-ml samples of the dilutions were used for the assay of alkaline phosphatase with *p*-nitrophenyl phosphate, as described previously for blood serum (Slein and Logan, 1960). Phosphatase samples from homogenate controls that had been incubated without PF or with an excess of standard crude PF (about 300 units) were incubated for exactly 4 min at 37 C. One unit of PF is the amount that releases sufficient alkaline phosphatase from a homogenate sample to produce an absorbancy of 0.1 in the phosphatase test in 12 min with a 1-cm light path at 395 mμ. Other phosphatase samples from the homogenate treated with various phospholipase fractions were incubated for 12 min along with samples from controls that had been incubated without PF or with 3 to 6 units of standard PF. The absorbancies were corrected for alkaline phosphatase released spontaneously from the control homogenates that had been incubated without PF. The homogenate samples treated with excess standard crude PF served to determine the maximal amount of phosphatase that could be released from each preparation. All of the supernatant solutions were diluted so that the absorbancies obtained in the phosphatase test were not greater than about 1.3, which is within the range of proportionality for the *p*-nitrophenyl phosphate released. Phosphatase released and the amount of PF added were proportional if the amount of phosphatase was not greater than one-third of maximum found with excess PF. The 4-min absorbancy values obtained with excess PF were multiplied by three to compare the 12-min samples with them.

Although the absolute amount of phosphatase released varied from one homogenate to another, the values obtained with any given PF fraction relative to the standard PF were always very nearly the same. The activity of the standard crude PF solution was stable when stored frozen for over a year even at a concentration of only 0.1 mg of protein per ml of Ringer phosphate solution (*p*H 7.5). To compare the relative PF activities of the phospholipase fractions, the standard crude PF was given an arbitrary value of 30 units per μg of protein, i.e., 0.1 μg released phosphatase from a homogenate so that the corrected absorbancy in the phosphatase test was 0.3. For each new homogenate preparation and supernatant fluid dilution used for phosphatase determination, a factor was calculated to relate unknown PF samples to the standard crude PF.

The sensitivity of the kidney cortex homogenate test for measuring PF is of the same order of magnitude as the previously used bone slice procedure. Because of the very high PF activity of certain phospholipase fractions, it was necessary to dilute them to less than 1 μg of protein per ml. In such cases, serum albumin (0.1 mg/ml of Ringer phosphate) was included to stabilize the PF from inactivation. Although the kidney alkaline phosphatase released by PF was very stable at 5 C, the activities were always measured on the day that the homogenates had been treated with PF. The same washed homogenate preparation was used for tests in the morning and afternoon of the day of preparation; the spontaneous leakage of phosphatase increased during storage in ice, but the correction was not excessive. Breakdown of cells during storage of intact cortex in cold Ringer phosphate solution appeared to be greater than with the homogenate, so that it was better to use the same homogenate in tests over a 6-hr period than to prepare fresh homogenates from a given sample of kidney cortex.

Characterization of phospholipases by the use of quantitative paper chromatography. We had hoped to measure the degradation of purified phospholipids individually by the various phospholipase fractions of *B. cereus*, but this was not feasible, perhaps partly because of an unfavorable surface charge distribution with aqueous emulsions of single phospholipids (Bangham and Dawson, 1962). A compromise test system was used in which a mixture of phospholipids was incubated with the phospholipases, and degradation was measured quantitatively after elution of the stained phospholipid spots from chromatograms. Most of the results were obtained with an emulsion of "purified" soybean lecithin (Mann Research Laboratories, Inc., New York, N.Y.) supplemented with sphingomyelin (SPH) (General Biochemicals, Chagrin Falls, Ohio), and in some cases with lysolecithin (lyso PTC) (General Biochemicals). The soybean "lecithin" contained significant amounts of phosphatidyl ethanolamine (PTE) and phosphatidyl inositol (PTI) as well as phosphatidyl choline (PTC, lecithin). It also was contaminated with what appeared to be phosphatidic acid, smaller amounts of lyso PTC, and other substances. Approximately 60% of the phos-

phorus was accounted for as PTC, PTE, PTI, and lyso PTC. An emulsion containing 100 mg of soybean "lecithin" per ml of distilled water was prepared by treatment for 10 min in a 10-kc Raytheon sonic oscillator while cold fluid was circulated around the chamber to prevent excessive heating. The emulsion was adjusted to about *p*H 7 by the addition of KOH. For tests with phospholipases, the emulsion was buffered with 0.133 M phosphate. When SPH was to be included, an emulsion was prepared by adding 40 mg of SPH to 2 ml of the "lecithin" emulsion and 4 ml of 0.2 M phosphate buffer (*p*H 7.0). Emulsions were stored at 5 C with 1:10,000 Merthiolate as preservative.

To 0.15 ml of the buffered emulsion in 7.5 × 75-mm tubes was added 0.15 ml of phospholipase or distilled water. The tubes were stoppered, incubated at 37 C for various times, and chilled in ice for 1 min. Duplicate 10-μliter samples were placed on Whatman no. 3MM paper that had been impregnated with silicic acid, and the chromatograms were developed for 3.5 hr at about 24 C with diisobutyl ketone-glacial acetic acid-water (40:20:3, v/v), essentially as described by Marinetti (1962). The papers were dried for 10 min in a forced-air drying oven at about 50 C. The strips were cut in two lengthwise, and the duplicate pairs were stained for 1 hr by immersion in acid fuchsin or brilliant green solutions in tubes 2.5 × 20 cm, one pair per tube. The stained chromatograms were blotted between facial tissue papers and rinsed, with gentle agitation at intervals, for 10 min each in two changes of rinsing solutions. After a final blotting, the strips stained with acid fuchsin were dried for 10 min at about 50 C, and those stained with brilliant green were only partly dried at about 35 C or less. Areas slightly larger than the spots stained red with acid fuchsin (PTE, PTC, lyso PTC, and SPH) were cut out with scissors and further cut into pieces about 4-mm square that were allowed to stand overnight at about 24 C with 2 ml of tertiary butanol-0.2 M acetate buffer, *p*H 5.0 (1:1, v/v) in stoppered tubes (1 × 10 cm). PTI spots were stained blue with brilliant green and were eluted in the same way with 3 ml of the solution. Blank areas corresponding in size to the various samples were also eluted to correct for the background staining that was slight with acid fuchsin but was more significant in the case of brilliant green. After elution, the paper bits were stirred with a glass rod, and the samples were centrifuged at about 2,000 × *g*. The absorbancies of the supernatant solutions were measured at 640 mμ (maximum for the brilliant green stain under these conditions) or at 560 mμ (acid fuchsin) in a Beckman DU spectrophotometer with the eluting fluid as reference solution and a 1-cm light path. The values were corrected for the blanks, and the percentage degradation of each phospholipid was determined by comparing the absorbancies with those obtained with control samples that had been incubated without phospholipase.

Phosphate buffer (*p*H 7.0) was selected for use after preliminary tests indicated that the degradation of phospholipids was better under these conditions than with other buffers or *p*H values tried. Strong buffering is needed to prevent a decrease in *p*H. The milkiness that usually develops in the reaction mixture during phosphilipase action tends to become flocculent if the *p*H decreases, and uniform sampling with a micropipette becomes impossible. Adequate activity was obtained with the commercial "lecithin" without added metal cofactors; therefore, these were omitted from the reaction mixture, since the tendency for flocculation seemed greater with added metal ions.

The procedure for the quantitative analysis of phospholipids is based on an elegant series of papers (Bungenberg de Jong and van Someren, 1959; Hooghwinkel, Lexmond, and Bungenberg de Jong, 1959; Hooghwinkel and van Niekerk, 1960; Bungenberg de Jong, 1961). Because of its availability, we used acid fuchsin instead of Edicol Supra Ponceau 4 RS or other Ponceau dyes that also gave less intense colors. The acid fuchsin was certified reagent quality obtained from Fisher Scientific Co., Pittsburgh, Pa. (C.I. no. 42685). The dye solution consisted of 0.02% acid fuchsin in 0.2% $UO_2(NO_3)_2 \cdot 6H_2O$, 0.01 N HCl. The solution for rinsing out excess acid fuchsin was the same, but with the dye omitted. The brilliant green was certified and obtained from the National Aniline Division of the Allied Chemical and Dye Corp. (C.I. no. 662). It was used as a 0.01% solution in 0.01 N HCl after aging at about 24 C for 16 hr or more. Excess brilliant green was rinsed from chromatograms with 0.1 M acetic acid. The *p*H 5 solution that was described above for eluting both stains from chromatograms was developed because brilliant green was destroyed by the more acid solvent recommended for acid fuchsin or the Ponceau dyes (Hooghwinkel and van Niekerk, 1960). Acid fuchsin did not visibly stain PTI, but brilliant green, which was strongly bound by the acidic PTI, also gave faint blue spots with PTE, PTC, lyso PTC, SPH, and phosphatidyl serine (PTS). In our tests, only SPH was of concern, since it partly overlapped PTI on chromatograms. However, the color contributed by SPH was insignificant when compared with that given by PTI in our samples, so that it was possible to measure PTI by staining with brilliant green. The blue color obtained with PTI approached an end point that was about 25% of the control value under most conditions of testing with the phospholipases. For this reason, a decrease of 75% from the absorbancy of the control sample was assumed to represent complete degradation of PTI in our samples, and the values were adjusted accordingly. The absorbancy was proportional to concentration when 5-, 10-, and 15-μliter samples of the phospholipids were separated by paper chromatography.

The acid-soluble products split from the phospholipids were obtained by stopping enzyme ac-

tion by adding trichloroacetic acid to give a final concentration of 5%. After chilling in ice for 15 min, the precipitate was removed by filtration through Schleicher and Schuell no. 602 ED paper. When phosphorus determinations were to be made, the phosphate buffer of the reaction mixture was replaced by 2,4,6-trimethylpyridine buffer. The acid-soluble products were separated without further treatment by descending chromatography on Whatman no. 1 paper for 6 hr at about 24 C with the isopropanol-glacial acetic acid-water (3:1:1, v/v) solvent of Kerr and Kfoury (1962). The phosphorus-containing spots were made visible by spraying with the Hanes and Isherwood reagent (1949), heating for 1 min at about 85 C, and then exposing the paper to ultraviolet radiation (Bandurski and Axelrod, 1951). The spots were cut out, ashed for about 15 min at about 200 C until the paper was digested and the sample bleached, and phosphorus was determined according to the procedure of Gerlach and Deuticke (1963). Total phosphorus in the acid filtrates was determined by the method of Fiske and SubbaRow (1925) or by the procedure of Gerlach and Deuticke (1963). It was not possible to measure inorganic orthophosphate directly in the acid filtrates, because a precipitate formed during color development. Therefore, it was estimated after separation on Whatman no. 1 paper with a solvent described by Wood (1961) [isopropyl ether–*n*-butanol–90% (w/v) formic acid (30:30:20, v/v)]. The chromatography was carried out with the solvent descending for 16 hr at about 24 C.

Inositol analysis. The occurrence of PTI in the soybean "lecithin" was suspected on the basis of its R_F value and staining properties with Rhodamine 6 G (Marinetti, 1962), as well as with brilliant green (Bungenberg de Jong, 1961). Chemical methods for the analysis of inositol are not specific, and biological assays are not sufficiently sensitive. A specific method that uses inositol dehydrogenase isolated from *Aerobacter aerogenes* grown with inositol as carbon source was reported by Weissbach (1958). With the crude extract as a source of enzyme, the method is not reliable, because reoxidation of the reduced nicotinamide adenine dinucleotide ($NADH_2$) occurs and prevents the attainment of a stable end point. This difficulty was overcome by using the procedure described by Larner et al. (1956) modified in the following way. Although partial purification of the dehydrogenase was reported by Larner et al. (1956) and Larner (1962), we obtained good proportionality between inositol concentration and absorbancy even with a crude extract. A mucoid strain of *A. aerogenes* was kindly supplied by R. A. Altenbern of Fort Detrick. The cells were grown as described by Magasanik (1953) and were harvested by centrifugation at about 38,000 × *g* at 2 C. The cells were washed twice with cold distilled water and were suspended with cold 0.05 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.5, to give approximately 20 g of cells (wet weight) per 40 ml of suspension. The cells were disrupted for 20 min at 1.25 amp in a 10-kc Raytheon sonic oscillator cooled with fluid circulating at about −5 C. The material was centrifuged for 30 min at about 38,000 × *g* at 2 C, and the slightly opalescent supernatant fluid containing about 9 mg of protein per ml was stored frozen. Samples containing 0.1 and 0.2 μmole of *myo*-inositol and samples with neutralized hydrolyzates were prepared along with a control without inositol and another without inositol, NAD, or enzyme. The samples were made up to 1 ml with final concentrations of 0.04 M Tris (*p*H 8.6), 0.025 M $K_3Fe(CN)_6$, and 0.002 M NAD, and were incubated at 37 C for a time determined to give maximal Prussian blue color development and absorbancies proportional to the two concentrations of inositol. An incubation of 15 to 30 min was sufficient with 0.9 mg of protein from one crude extract that we prepared. The reaction was stopped by the addition of 0.1 ml of 100% (w/v) trichloroacetic acid. After the addition of 1.7 ml of water and 0.2 ml of Duponol-iron reagent (Larner et al., 1956), the samples were mixed and read in a Beckman DU spectrophotometer at 560 mμ within about 15 min. The control without added inositol, NAD, or enzyme served as the reference solution. The absorbancy was doubled at 620 mμ and tripled at 700 mμ, so that these wavelengths might be used to increase the sensitivity. Although the absorbancies continued to increase with time, the values, corrected for the control without added inositol, remained fairly constant, especially at 560 mμ (an absorbancy of about 0.1 per 0.1 μmole of *myo*-inositol).

Since inositol dehydrogenase will act only on free inositol, it is necessary to hydrolyze PTI before analysis. When the inositol content of a spot on a chromatogram was desired, the area was cut out and eluted repeatedly with chloroform-methanol (7:3, v/v); the eluates were dried in a Flash-Evaporator and transferred with a small amount of the solvent to a tube (7.5 × 75 mm) for drying. After the addition of 0.1 ml of 6 N HCl, the end of the tube was sealed off, and the sample was hydrolyzed for 6 hr at 110 C (Böhm and Richarz, 1954). The hydrolysate was neutralized with a measured volume of NaOH. The sample was centrifuged and the supernatant solution was analyzed for inositol. Since the completion of this work, a fluorometric micromethod for the enzymatic determination of inositol has been described (Garcia-Buñuel and Garcia-Buñuel, 1964).

Extraction of phospholipids from cells. The procedure of Bligh and Dyer (1959) was used to extract phospholipids from aqueous suspensions of washed erythrocytes or from rabbit kidney cortex homogenates. Relatively small volumes of materials were extracted in a VirTis 45 homogenizer at low speed or in a Waring Blendor with the speed reduced by means of a rheostat to avoid excessive splattering. Homogenate (9 ml) was extracted and centrifuged briefly to clump the particles and facilitate filtration. After filtration, the mixture

was centrifuged for 10 min, and the liquid phases were separated with practically no film at the interface. The upper layer was carefully removed by aspiration through a fine-tipped pipette so that essentially no loss of the lower chloroform layer occurred. The latter was dried within a few minutes at 45 C in a Flash-Evaporator with the condenser in an ice bath. The residue was carefully dissolved with 0.5 ml of isoamyl alcohol-benzene (1:1, v/v) and was transferred by pipette to a small tube for storage at −15 C.

Practically all of the phospholipids of mature mammalian erythrocytes are located in the cell membrane (Ways and Hanahan, 1964). Therefore it is possible to measure the phospholipid content of the membranes by the use of whole erythrocytes. Erythrocytes were washed three to four times with isotonic NaCl; leukocytes were removed by aspiration after each centrifugation. The packed cells were suspended with an equal volume of Ringer phosphate solution (*p*H 7.5), and as little as 6 ml was used for the extraction of phospholipids. It was not necessary to centrifuge the suspension of extracted erythrocytes before filtration. After filtration, the mixture was centrifuged, and the upper layer, with as much interfacial precipitate as possible, was carefully removed by aspiration. The chloroform layer was dried, and the residue was dissolved, as described above for kidney.

Hemolysin activity. Sheep and rabbit erythrocytes were washed and leukocytes were removed, as described in the preceding paragraph. The packed cells were diluted 1:40 with Ringer phosphate (*p*H 7.5). To 4.8 ml of the diluted erythrocytes in a 12-ml centrifuge tube at 37 C was added 0.2 ml of the solution to be tested for hemolysin activity. The tube was closed with a Saran-wrapped stopper; the contents were mixed several times by inversion and incubated at 37 C for 30 min. The tube was quickly chilled in ice, and was centrifuged at 5 C; 1 ml of the supernatant fluid was mixed with 1 ml of Drabkin's reagent. After 10 min, the cyanmethemoglobin color was read at 540 mμ in a Beckman DU spectrophotometer with Drabkin's reagent and diluted with an equal volume of water, as the reference solution (Wintrobe, 1956). Readings were corrected for slight spontaneous hemolysis of control samples. Complete hemolysis resulted in absorbancies of about 2.0 to 2.5 in control samples with excess crude PF. When the destruction of hemolysin activity by trypsin was studied, the percentage of residual activity was calculated from a curve obtained with graded amounts of untreated hemolysin.

The relationship between hemolysis and the phospholipid content of erythrocyte membranes was studied by suspending the packed washed cells with an equal volume of Ringer phosphate (*p*H 7.5) and incubating with phospholipase at 37 C. After incubation, 0.2-ml samples were diluted with 3.8 ml of Ringer phosphate or water, centrifuged, and 1 ml of each supernatant fluid was mixed with 1 ml of Drabkin's reagent for measuring hemolysis. The sample that had been diluted with water gave the value for complete hemolysis. The bulk of the phospholipase-treated erythrocyte suspension was used for the extraction and analysis of phospholipids.

Treatment with trypsin. Crystalline trypsin was obtained from Worthington Biochemical Corp., Freehold, N.J. Soybean trypsin inhibitor (STI) was a product of the Armour Laboratories. The trypsin was dissolved in distilled water to give a solution of 2 mg/ml. The same concentration of STI was preapred with 0.01 M Tris (*p*H 7.5). Phospholipase fractions were treated with approximately 0.1 mg of trypsin per ml in 0.02 M Tris (*p*H 7.1) for various times at 37 C. The trypsin activity was stopped by adding an equal weight of STI. Control samples were incubated with STI added *before* the trypsin.

Preparation of lysophosphatides. Approximately 100 mg of commercial soybean "lecithin" were incubated with 0.5 mg of *Crotalus adamanteus* venom (Sigma Chemical Co., St. Louis, Mo.) for 2 hr at 37 C as an emulsion buffered with 0.05 M phosphate (*p*H 7.0). Most of the PTC and PTE of the "lecithin" was converted to the corresponding lysophosphatides.

RESULTS

Enzymatic degradation of phospholipids. Tests with crude PF preparations, such as the protamine-treated ammonium sulfate precipitate described previously (Slein and Logan, 1962), showed that PTE, PTC, PTI, and SPH were readily degraded. The effect on lyso PTC was much less marked, if at all significant. Since the commercial soybean "lecithin" contained too little lyso PTC for accurate testing, it was supplemented with a commercial product. A mixture produced by the action of snake venom on soybean "lecithin" contained both lyso PTE and lyso PTC (see Materials and Methods). Neither of these lysophosphatides appeared to be significantly affected by crude PF, although the residual parent compounds and PTI were degraded by the PF. The degradation of PTI, PTC, PTE, and SPH by various amonts of crude PF preparation in 30 min is shown in Fig. 1. Because of the complexity of the test system and the analytical procedure, the activities of various fractions (see below) were merely compared by calculating the percentage of degradations obtained under conditions that led to partial decomposition of the various phospholipids, and no activity units have been defined. Since activities were not linearly proportional to enzyme concentration under these conditions, it was necessary to construct curves like those in Fig. 1 with intact enzymes to calculate the percentage of inactivation caused by trypsin.

The PTI in the commercial "lecithin" was identified by analyzing the brilliant green-stained

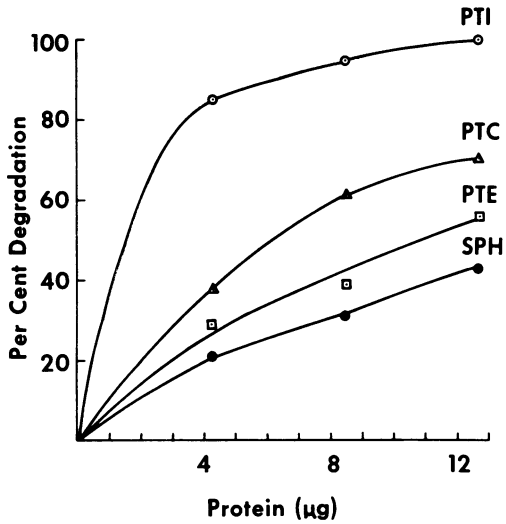

FIG. 1. *Degradation of phospholipids in an emulsion by different amounts of a crude phospholipase preparation in 30 min at pH 7 and 37 C. For abbreviations, see footnote of Table 1.*

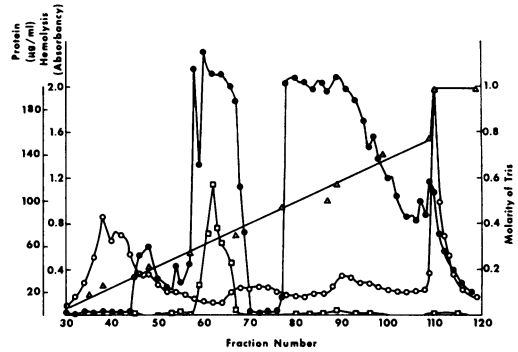

FIG. 2. *Distribution of protein and of sheep and rabbit hemolysins in fractions obtained by the elution of a crude preparation of phosphatasemia factor from a DEAE cellulose column with increasing concentrations of Tris buffer (pH 8.6). Symbols:* △ = *molarity of Tris;* ○ = *protein concentration;* □ = *rabbit hemolysin;* ● = *sheep hemolysin.*

material after it was eluted from a paper chromatogram. The mono PTI spot accounted for about 50% of the total inositol found in the directly analyzed "lecithin" emulsion. A second inositol-containing spot, which was assumed to be a diphosphatide, had a lower R_F than the principal PTI spot. Approximately 6 mg of mono PTI were present in 1 ml of the "lecithin" emulsion that contained 100 mg (dry weight).

The acid-soluble phosphorus compounds accounted for practically all of the phospholipids degraded by the phospholipases in crude PF. For example, soybean "lecithin" was incubated with crude PF for 1 hr so that 90 to 98% of the PTI, PTE, and PTC were degraded. Direct analysis of the trichloroacetic acid filtrates showed that 1.688 mg of acid-soluble phosphorus had been released from 1 ml of emulsion. Analysis of the phosphoryl choline, phosphoryl ethanolamine, and phosphoryl inositol products from paper chromatograms yielded 1.754 mg of phosphorus. Thus, 104% of the acid-soluble phosphorus released was recovered in the products that represented the principal phospholipids of the "lecithin" emulsion. When another preparation of PF was incubated with soybean "lecithin" supplemented with SPH, 89% of the acid-soluble phosphorus released was accounted for by the same products. In either case, inorganic orthophosphate accounted for only about 2% of the total acid-soluble phosphorus released. These results indicate that the phospholipase of culture filtrates of *B. cereus* liberate diglycerides and acid-soluble phosphate esters from the phosphatides, and phosphoryl choline from SPH. Very little phosphomonoesterase activity is present in crude PF under these conditions at *p*H 7. Other tests have shown that crude PF also has no significant alkaline phosphatase activity at *p*H 10.5 with *p*-nitrophenyl phosphate as substrate. The fact that no lysophosphatides were ever found to accumulate on chromatograms after incubation of phospholipids with crude PF or any of the fractions, although the lysophosphatides were not significantly degraded by these preparations, indicates that no significant "phospholipase A" activity exists in them.

A preparation containing 19 mg of crude PF protein was fractionated on *N*,*N*′-diethylaminoethyl (DEAE) cellulose as described previously (Slein and Logan, 1963). Approximately 80% of the protein was recovered in 119 fractions of about 5 ml each (Fig. 2). Protein was concencentrated in two principal regions: fractions 30 to 50 and fractions 109 to 115. The latter sharp peak resulted from the sudden increase of Tris concentration from about 0.8 to 1 M to elute most of the residual protein from the column. Very little phospholipase activity was found below fraction 30. After a preliminary survey, fractions were selected for further tests to determine their relative phospholipase activities. The results are presented in Table 1. The original crude PF attacked all four phospholipids. The separation of three discrete activities is indicated by the data. Fractions 35 to 50, which were eluted by 0.1 to 0.2 M Tris (*p*H 8.6), degraded PTE and PTC. It is not clear whether one or two enzymes are involved in the degradation of these two phospholipids. A phospholipase that attacked SPH began to ap-

TABLE 1. *Relative phospholipase activities of fractions obtained by elution from a DEAE cellulose column with increasing concentrations of Tris buffer (pH 8.6)*

Fraction no.*	Amt of protein tested	Per cent degradation†			
		PTE	PTC	SPH	PTI
	μg				
Original material	23.6	46	66	56	100
38	17.1	29	26	0	0
44	10.6	20	17	0	0
46	7.3	15	18	0	0
51	4.8	12	7	10	0
57	3.3	0	0	35	16
62	2.2	0	0	55	29
66	4.0	0	0	51	52
72	4.9	0	0	26	83
80	3.4	0	0	6	97
100	4.3	0	0	9	55
110	39.4	0	0	7	61

* See Fig. 2.

† Samples were incubated for 1 hr at 37 C. PTE = phosphatidyl ethanolamine, PTC = phosphatidyl choline, SPH = sphingomyelin, and PTI = phosphatidyl inositol.

pear at about fraction 50 and had highest activity in fraction 62. The enzyme that degraded PTI overlapped the preceding, but was concentrated in fractions 70 to 80 that were eluted by about 0.4 M Tris. A similar distribution of activities was obtained with two other fractionations of crude PF.

Hemolysin activity. The data in Fig. 2 show the distribution of hemolysin activities as measured with sheep and rabbit erythrocytes. No significant hemolysin activity was detected among the first 29 fractions that were omitted from the figure. No attempt was made to determine specific activities, and the results merely indicate the absorbancies obtained after the incubation of erythrocytes for 30 min at 37 C with 0.1 ml of each fraction. In some cases, excess hemolysin may have been present when hemolysis was essentially complete (absorbancies of about 2.0). Sheep erythrocyte hemolysin appeared in two broad series of fractions, whereas rabbit erythrocyte hemolysin activity was found in one region that coincided roughly with one of the principal sheep hemolysins. Approximately 1 μg of protein of fraction 62 was sufficient to produce the marked hemolysis noted in Fig. 2.

Changes in the two major phospholipid components of sheep erythrocyte membranes that occurred during partial hemolysis by crude phospholipase preparations are presented in Table 2.

TABLE 2. *Loss of phospholipids from sheep erythrocyte membranes during partial hemolysis by crude phospholipase*

Expt*	Protein†	Time	Hemolysis	Per cent degradation‡	
				PTE	SPH
	μg	min	%		
1.....	6.8 A	30	34	15	9
	None	30	5	—	—
2.....	68 A	30	61	40	33
	68 A	60	71	65	62
	None	60	0.3	—	—
3.....	14.2 B	15	32	18	13
	14.2 B	60	42	34	36
	None	60	1	—	—

* In experiments 1 and 2, the volume of washed erythrocyte suspension was 25 ml; in experiment 3 it was only 6 ml.

† A and B were different preparations of crude phospholipase.

‡ For abbreviations, see footnote of Table 1.

Both preparations were able to degrade PTC, PTE, PTI, and SPH in emulsions of the phospholipids. Increasing the concentration of phospholipase 10-fold in experiment 2 as compared with experiment 1 increased the degradation of phospholipids about threefold to fourfold, but hemolysis was not quite doubled. Incubation for a second 30-min period in experiment 2 resulted in a much greater degradation of phospholipids relative to the increase in hemolysis. Similarly, with another preparation in experiment 3, a small change in hemolysis was accompanied by much greater phospholipid destruction.

Prior to testing the sensitivity of hemolysin to trypsin, the effect of soybean "lecithin" on hemolysin activity was studied for possible use as an agent to protect against trypsin in the way that PF was protected (Slein and Logan, 1963). However, hemolysin was found to be markedly inhibited by such low concentrations of "lecithin" that the latter could not be used as a protective agent. The inhibition by "lecithin" is shown in Table 3. When 1 μg of crude phospholipase protein was mixed with "lecithin" at 0 C *immediately* before adding to the erythrocytes (experiment 1), the inhibition of hemolysin activity decreased with decreasing concentrations of "lecithin" and was not complete even with 100 μg. In experiment 2, however, incubation of the phospholipase with only 7 μg of "lecithin" for 15 min at 37 C before adding it to the red cells inhibited hemolysis completely. Inhibition was marked even after 20 min at 0 C.

TABLE 3. *Effect of soybean "lecithin" on the hemolysis of sheep erythrocytes by crude phospholipase*[a]

Expt	Incubation[b]	"Lecithin"[c]	Hemolysis
		μg	%
1....	None	250	1.2
	None	100	6.9
	None	50	11.0
	None	40	13.5
	None	30	15.5
	None	20	18.6
	None	10	28.9
	None	0	37.6
	None	0[d]	0.8
2....	15 min 37 C	7	0.8
	15 min 37 C	0	27.1
	20 min 0 C	7	6.4
	20 min 0 C	0	28.1
	None	7	21.5
	None	0	31.2
	None	0[d]	0.8

[a] In each case (except for the controls) 1 μg of crude PF protein was present during hemolysis for 30 min at 37 C.

[b] PF was incubated with "lecithin" before adding to the erythrocyte suspension. None = PF was mixed with "lecithin" at 0 C *immediately* before adding to the erythrocytes.

[c] Besides PTC, the "lecithin" contained PTE, PTI, and other contaminants (see Materials and Methods). For abbreviations, see footnote of Table 1.

[d] Control sample without PF.

Effect of PF fractions on rabbit bone and kidney cortex homogenates. The phospholipase that was easily eluted from DEAE cellulose had no PF activity and inhibited the PF activity of a fraction eluted by about 0.4 M Tris when tested with the bone slice assay method (Slein and Logan, 1963). Unlike the results with bone slices and similar results obtained with bone homogenates, either fraction was able to release phosphatase from kidney cortex homogenates. Furthermore, when both fractions were present with the kidney homogenate, the release of alkaline phosphatase was stimulated rather than inhibited. A comparison of results obtained with the two rabbit tissues is shown in Table 4. The fraction A phospholipase was a combination of fractions eluted from DEAE cellulose by low concentrations of Tris and degraded only PTC and PTE in our phospholipase tests. The PF was a crude preparation. With the bone homogenate, as had also been noted with bone slices, fraction A inhibited the spontaneous leakage of phosphatase (negative corrected value) as well as PF activity. With the kidney homogenate, either material caused the release of phosphatase, but the combination resulted in a stimulation twice that expected from the sum of the effects of each alone. This is distinct from the proportionality of phosphatase release to PF concentration that is obtained with preparations that have high PF activity (see fractions 72 and 80 in Table 6).

TABLE 4. *Effect of the phospholipase that is readily eluted from DEAE cellulose (fraction A) on the release of alkaline phosphatase from rabbit epiphyseal bone and kidney cortex homogenates by crude phosphatasemia factor (PF)*

Homogenate	Additions	Alkaline phosphatase (absorbancy)*
Bone.....	PF	0.519
	Fraction A	−0.115
	Both	0.203
Kidney.....	PF	0.345
	Fraction A	0.275
	Both	1.261

* Average of duplicate values corrected for the spontaneous release of phosphatase that occurred in the absence of PF or fraction A.

TABLE 5. *Relative phosphatasemia factor (PF) activities of fractions obtained by elution from a DEAE cellulose column with increasing concentrations of Tris buffer (pH 8.6)*

Fraction no.*	PF activity† (units/μg of protein)
Original material	98.5
38	0.17
44	0.44
46	0.52
51	0.66
57	2.46
62	4.60
66	7.97
72	302.0
80	1338.0
100	71.2
110	10.6

* See Fig. 2.

† Average of two or more determinations made with rabbit kidney cortex homogenates.

Kidney cortex homogenates were used for comparing the PF activities of the same fractions for which the phospholipase activities are given in Table 1. The results are presented in Table 5. Of the fractions tested, PF activity was most highly concentrated in fraction 80, which also had the greatest PTI-phospholipase activity (Table 1). The effects of combinations of certain

TABLE 6. *Effect of combinations of phospholipase fractions on the release of alkaline phosphatase from rabbit kidney cortex homogenates*

Expt	Fraction	Protein	Phosphatase released (absorbancy)*
		μg	
1.....	38	8.55	0.110
	38	17.10	0.074
	72	0.0075	0.335
	72	0.015	0.664
	38	8.55	
	plus		1.006
	72	0.0075	
2.....	38	8.55	0.182
	80	0.0015	0.271
	80	0.030	0.531
	38	8.55	
	plus		0.819
	80	0.0015	

* Values corrected for the spontaneous release of alkaline phosphatase that occurred in the absence of added phospholipase.

fractions on the PF activity with kidney homogenates are shown in Table 6. The low PF activity and lack of proportionality with two concentrations of fraction 38 are typical of results obtained with the phospholipase that is easily eluted from DEAE cellulose, in contrast to material having high PF activity, such as fraction 72 or 80. Only a few millimicrograms of the proteins of fractions 72 and 80 were needed for a marked release of alkaline phosphatase from kidney cortex cells. Combinations of fraction 38 with fraction 72 or 80 resulted in approximately twofold stimulations of phosphatase release over that expected from the sum of the effects of each alone.

The presence of 1 mg of commercial soybean "lecithin" in 0.7 ml of the kidney homogenate reaction mixture completely inhibited the release of alkaline phosphatase by a crude PF preparation. This agrees with the effect reported for the bone slice test system (Slein and Logan, 1963).

A few tests were made to relate the liberation of phosphatase and the degradation of phospholipids naturally present in kidney cortex homogenates by phospholipases. A combination of several fractions eluted from DEAE cellulose by 0.05 M Tris (fraction A) was compared with a combination eluted by 0.4 M Tris (fraction D). Although fraction D released about 10 times as much phosphatase as did fraction A, the latter degraded practically all of the PTE and PTC in the homogenate, whereas fraction D degraded only PTI significantly. In another test with crude PF, analysis for inositol revealed that the phospholipid extract of the control homogenate contained about 2.8 μmoles of inositol per ml of phospholipids, whereas the sample treated with PF had only 0.8 μmole. These results might be interpreted to indicate that the degradation of a minor phospholipid component (PTI) in the kidney cell membrane is more critical for the damage that results in the release of alkaline phosphatase than is the degradation of PTE and PTC. However, unlike the mature erythrocytes, kidney cells have phospholipids in components other than the external membranes (e.g., mitochondria) so that no correlation between membrane damage and phospholipid degradation can be made with certainty until methods for the isolation of mammalian cell membranes are available.

Effect of trypsin on the enzymatic activities of combined phospholipase fractions. To have sufficient material for analysis, it was necessary to combine the residues of groups of fractions, concentrate them in Carbowax (polyethylene glycol), and dialyze against 0.01 M Tris (*p*H 7.5) at 5 C. Groups with similar activities were selected for combination as summarized in Table 7. The distribution of activities corresponded, in general, to that expected from tests with individual fractions as shown in Fig. 2 and in Tables 1 and 5. The hemolysin activities of fraction III in Table 7 were obtained with 8.2 μg of protein, whereas no significant hemolysis occurred with about 2 μg of fractions 70 to 76 when originally tested (Fig. 2).

The effects of trypsin on the various activities are presented in Table 8. Control tests with equivalent amounts of trypsin plus STI were made to be sure that these substances themselves did not have any of the activities to be measured. Neither the phospholipase activity of fraction I nor its slight PF activity was significantly inactivated by trypsin. The phospholipase that degraded SPH also seemed to be relatively resistant to trypsin. PTI-phospholipase and PF activities were rather sensitive to inactivation by trypsin. Fraction II hemolysin was also relatively sensitive to trypsin, but the other fractions had intermediate values.

DISCUSSION

The results obtained with preparations from culture filtrates of *B. cereus* have been duplicated in most respects with *B. anthracis*, strain Sterne (Sterne, 1937). The phospholipase activity is weaker in culture filtrates of *B. anthracis* (Slein and Logan, 1962), but fractionation of the crude material separates the various phospholipase activities in a manner entirely analogous to that obtained with *B. cereus*. The interaction between fractions in the bone slice and kidney homogenate tests for PF is also the same for the two species.

TABLE 7. *Enzymatic activities of groups of fractions that were combined after separation on a DEAE cellulose column*

Fraction[a]	Hemolysin[b]		Per cent degradation of phospholipids[c]					PF[d] activity (units/μg of protein)
	Sheep	Rabbit	Protein tested	PTE	PTC	SPH	PTI	
			μg					
Original material	0.530 (2.84)	0.514 (18.9)	23.6	46	66	56	100	98.5
I (37–43)	0.005 (19)	0.009 (38.5)	38.5	46	66	0	9	0.22
II (59–66)	1.309 (1.0)	1.223 (2.14)	7.13	0	6	58	47	7.2
III (70–76)	0.219 (8.2)	0.814 (8.2)	1.0	0	0	17	80	1170
IV (80–86)	0.760 (0.6)	0.085 (5.9)	1.18	0	0	19	81	936

[a] Numbers in parentheses represent the fractions in Fig. 2 that were combined.
[b] Absorbancy values corrected for the slight spontaneous hemolysis of control samples. Complete hemolysis would be indicated by an absorbancy of about 2.0. The μg of protein tested are shown in parentheses.
[c] Samples were incubated for 1 hr at 37 C with the amounts of protein shown. For abbreviations, see footnote of Table 1.
[d] Phosphatasemia factor (PF) activity was determined with rabbit kidney cortex homogenates.

TABLE 8. *Effect of trypsin on enzymes present in groups of fractions that were combined after separation on a DEAE cellulose column*

Fraction[a]	Treatment with trypsin[b]	Hemolysin inactivation		Phospholipase[c] inactivation				PF[d] inactivation
		Sheep	Rabbit	PTE	PTC	SPH	PTI	
	min	%	%	%	%	%	%	%
Original material	30	40	34	7	13	16	70	83
I (37–43)	20	—[e]	—	0	0	—	—	0
	60	—	—	0	0	—	—	0
II (59–66)	30	80	80	—	—	11	72	>90
III (70–76)	15	39	56	—	—	27	84	97
IV (80–86)	15	52	—	—	—	18	81	100
	30	63	—	—	—	28	89	100

[a] Numbers in parentheses represent the fractions in Fig. 2 that were combined.
[b] Approximately 0.1 mg of trypsin per ml except for fraction I, which was treated with about 0.3 mg per ml.
[c] For abbreviations of phospholipids, see footnote of Table 1.
[d] Phosphatasemia factor (PF) measured with kidney cortex homogenates.
[e] Dashes indicate that activities were absent or were too low for accurate measurement and were not tested here (see Table 7).

It seems likely that the phospholipases may be part of the toxin complex of *B. anthracis* as well as that of *B. cereus*.

The "lecithinase" of *B. cereus* has been found to comprise a group of phospholipases that are able to split phosphoryl choline, phosphoryl ethanolamine, and phosphoryl inositol from the phospholipids PTC, SPH, PTE, and PTI. A possible lack of specificity has been ascribed to the "lecithinase" of *B. cereus* by others (Chu, 1949; Robinson, Harris, and Poole, 1957; Kushner and Feldman, 1958). We also noted slight activity with phosphatidyl serine (PTS), but this was not pursued further, because it was not possible to separate PTS from PTE sufficiently for accurate determination with the chromatographic procedure that we used. The trace amounts of PTS in the materials that we analyzed did not interfere significantly with the determination of PTE. The phospholipases that degrade PTI and SPH have been separated from those that attack PTE and PTC. The enrichment of

the PTI- and SPH-phospholipases in separate fractions, as well as their different susceptibilities to attack by trypsin, indicates that they are individual enzymes. We have not succeeded in separating the PTE- and PTC-splitting activities to any degree that would suggest the presence of two enzymes. The fact that these activities are both resistant to trypsin (Table 8) supports the idea that one phospholipase ("lecithinase") may degrade both substrates. The "lecithinase" of *B. anthracis* was also found to attack PTE and PTC (Costlow, 1958). The phospholipase preparations of *B. cereus* appeared to have little, if any, phosphomonoesterase or phospholipase A activities under our conditions of testing.

Hemolysin activity does not seem to be associated with the PTE- or PTC-phospholipase activity (fraction I, Table 7). This agrees with our previous conclusion with regard to the "inhibitor" phospholipase (Slein and Logan, 1963) and with the findings of Ottolenghi, Gollub, and Ulin (1961) that hemolysin could be separated from phospholipase. The separation of hemolysin and "egg yolk turbidity factor" was reported by Fossum (1963). Our results also indicate that the hemolysins are not identical with the SPH- or PTI-phospholipases. Although SPH-phospholipase was concentrated in fraction II that included fractions having good hemolysin activities for both sheep and rabbit erythrocytes (Fig. 2 and Table 7), the hemolysin activities appeared to be more sensitive to trypsin than did the SPH-phospholipase (Table 8). On the other hand, the PTI-phospholipase was generally more susceptible to inactivation by trypsin than were the hemolysins (fractions III and IV, Tables 7 and 8). Furthermore, fraction III had much more PTI-phospholipase activity than fraction II, but the latter had greater hemolysin activity (Table 7). Although fractions III and IV had rather similar SPH- and PTI-phospholipase and PF activities, fraction IV was much more hemolytic for sheep than for rabbit erythrocytes (Table 7). Other data in Table 1 and Fig. 2 support the independence of phospholipase and hemolysin activities.

In agreement with others, our chromatograms showed that the principal phospholipids of rabbit erythrocyte membranes are PTE, PTC, and SPH; those of sheep red cells are PTE and SPH (de Gier and van Deenen, 1961; Klibansky, Condrea, and de Vries, 1962). Hemolysin activity may be primarily caused by damage to some minor phospholipid or other constituent that we have not measured. The attack of membrane phospholipids by the phospholipases may occur secondarily after their exposure by the action of a true hemolysin in the manner reported for snake venoms (Condrea, de Vries, and Mager, 1964). The degradation of phospholipids in cells that have already hemolyzed may also account for the loss of phospholipids being relatively greater than the increases in hemolysis, as noted in Table 2. However, it is possible that degradation of the principal phospholipid components of the erythrocyte membrane contributes to hemolysis. The mechanism by which a crude soybean "lecithin" inhibits hemolysin activity is not known. The augmentation of the inhibition by incubation of the "lecithin" with the crude hemolysin indicates that phospholipase activity may be involved. Previous results have shown that "lecithin" markedly inhibited the PF activity on bone slices (Slein and Logan, 1963). This was interpreted as a competition between phospholipids of the "lecithin" emulsion and the natural substrates in the bone cell membrane for the site of the phospholipase responsible for PF activity. A similar inhibition of PF activity has been noted with kidney cortex homogenates.

PTE- and PTC-phospholipase activity does not seem to be essential for PF activity, although it stimulates the release of phosphatase from kidney cells by fractions that have relatively high PF activities. SPH-phospholipase also does not appear to be important for PF activity, since it was concentrated in fraction 62 that had relatively low PF activity (Tables 1 and 5), and, unlike PF, it was rather resistant to trypsin (Table 8). On the other hand, PTI-phospholipase was associated with high PF activity (Tables 1 and 5) and was sensitive to trypsin (Table 8).

The differences in the effects of various phospholipases on the release of alkaline phosphatase from bone and kidney cells are very interesting. The material that had only PTE- and PTC-phospholipase activity not only failed to liberate phosphatase from bone cells but also suppressed the rate of spontaneous leakage of phosphatase from the cells. Although it had slight PF activity with kidney cells, the activity tended to remain the same or to decrease slightly with increasing concentrations of the protein. This may be interpreted as an indication of the presence in the membranes of no, or relatively few, PTE and PTC molecules that are available to attack by the specific phospholipase(s) and that these sites may become saturated with the enzyme. With bone cells, the attachment of these phospholipase proteins may hinder the approach of other phospholipases to their specific phospholipid substrates that may be more important for the integrity of the membrane. On the other hand, such phospholipids as PTI may be readily available to the highly active PF phospholipase(s), or become so during degradation of both bone and kidney cell membranes, so that damage to the mem-

branes increases directly with the amount of PF added and results in a proportional increase in phosphatase liberation. In the case of the kidney cell, the two types of enzymes may not interfere with each other but may result in larger "holes" in the membrane, so that a stimulation of phosphatase release is obtained. Other explanations might be invoked, based on differences in specific activities of the phospholipases, differences in charge distributions of phospholipids in the cell membranes, etc.

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