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A Phospholipase in *Bacillus megaterium* Unique to Spores and Sporangia†

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ABSTRACT: A phospholipase activity which first appears in sporulating *Bacillus megaterium* is then found in mature, free spores. The enzyme is released from spores during germination or by mechanical disruption. The phospholipase is not essential for germination because it is destroyed by heating that does not affect the viability of the spore. The enzyme released from germinated spores behaves as a water-soluble enzyme and has been purified 170-fold to near homogeneity. It is characterized as specific in cleaving the 1-acyl linkage. The purified enzyme requires either a nonionic or anionic detergent for a negatively charged substrate, phosphatidylglycerol, but an anionic detergent (sodium taurocholate) for hydrolysis of neutral phospholipids. Thus the enzyme seems to prefer negatively charged substrate–detergent complexes. The phospholipase activity in sporangial extracts has properties similar to those of the purified spore enzyme, including A_1 specificity, pH and detergent responses, and the lack of any requirement for calcium or magnesium ions.

Phospholipid metabolism must play an important part in the membrane alterations involved in the development of the spore and in its germination. A fall of about 50% in the total phospholipid level during sporulation in *Bacillus megaterium* was found in an earlier study (Bertsch *et al.*, 1969; for a review of sporulation, see Kornberg *et al.*, 1968). We detected at about the same time in the sporulation process the appearance

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of a phospholipase activity which might be responsible for this drop in phospholipid level. We have studied this phospholipase not only for its possible importance in the sporulation process but also for the general role that phospholipases play in membrane metabolism. Furthermore, with an isolated phospholipase in hand, there is an opportunity to examine protein–phospholipid interactions as well as exploiting the enzyme for analysis and dissection of membranous structures.

We observed phospholipase activity in both sporulating cells and in mature spores. In this report we describe the purification of the spore phospholipase, the specificity of the enzyme in cleaving the 1-acyl linkage and the requirements for this cleavage. The activity in sporangial extracts had similar properties. Unlike the phospholipase A_1 activities found in *E. coli* (Proulx and Van Deenen, 1967; Scandella and Kornberg, 1971) and in animal cells (Gatt, 1968) which are firmly bound in the membrane, the spore enzyme has water-soluble characteristics. A water-soluble phospholipase A_1 has also been observed in rat liver lysosomes (Stoffel and Trabert, 1969; Franson *et al.*, 1971).

Experimental Section

A. Materials. Sources of materials were as follows: DEAE-cellulose, DE52, from Whatman; hydroxylapatite Bio-Gel HTP from Bio-Rad Laboratories; Polyethylene Glycol 6000 from Union Carbide; Bistris[1] and Hepes buffers from General Biochemicals; acrylamide and bisacrylamide from Eastman and subsequently recrystallized.

Solvent systems used in thin-layer chromatography (tlc) were (v/v): solvent A, chloroform–methanol–acetic acid–water, 100:50:14:6; solvent B, chloroform–methanol–acetic acid, 65:25:8; solvent C, chloroform–methanol–concentrated HCl, 87:13:0.5; and solvent D, petroleum ether–ether–formic acid, 60:40:1.6. Silica gel G for tlc was washed with methanol (Scandella and Kornberg, 1971).

The ^{32}P-labeled phospholipids, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), glucosaminylphosphatidylglycerol, and diphosphatidylglycerol, were extracted from *B. megaterium* cells grown about 30 min beyond the end of log phase, as described previously (Bertsch *et al.*, 1969). The compounds were purified by tlc with solvent B. 1-[^{32}P]Acyllysophosphatidylglycerol (lysoPG) was prepared by digestion of PG with snake venom (*Crotalus adamanteus*) and purified by tlc in solvent A. [^{32}P]Phosphatidic acid was prepared by digestion of PE with phospholipase D (cabbage) and purified by tlc on oxalic acid impregnated plates with solvent C. Semisynthetic [^{14}C]lecithin, labeled in the 2-acyl position with [1-^{14}C]palmitate, was a gift from Dr. C. J. Scandella.

Synthetic phospholipids were generously provided by Dr. P. P. M. Bonsen and Dr. A. J. Slotboom.

Bacillus megaterium was strain QM B1551.

B. Methods. 1. Growth of cells and spores. For sporangial preparations, cells were grown at 37° as previously (Bertsch *et al.*, 1969). Spores were grown at 30° essentially as described (Setlow and Kornberg, 1969). Growth of a 100-l. culture was in a Fermacell (New Brunswick Scientific Co.); the aeration rate was 2.8 l./sec. Spores were collected in a Sharples refrigerated continuous flow centrifuge and then washed twice with cold water. More extensive washing of the spores did not affect the specific activity of the enzyme in crude extracts. Washed spores resuspended in about 200 ml of water were lyophilized and yielded 50 g from the 100-l. culture.

2. Phospholipase from developing sporangia. Aliquots (30 ml) of a *B. megaterium* culture were taken at various times during the course of sporulation. Samples were centrifuged and the sporangia resuspended in 1.25 ml of buffer (0.1 M Tris-HCl, pH 7.4, 10 mM in β-mercaptoethanol). The suspensions were sonicated with 30-sec pulses of the Mullard sonicator until free spores were released, as determined by microscopy. The spores remained refractile. The sonicates were centrifuged for 10 min at 10,000*g* and the pellets discarded. The supernatant fluid, designated as sporangial extract, was assayed for phospholipase activity.

3. Germination of spores. A germination procedure which employs heating (*i.e.*, 10 min at 60°) as previously reported (Setlow and Kornberg, 1969) destroys the phospholipase activity. Germination by freezing and thawing does yield active enzyme. Lyophilized spores were suspended in 0.1 M NaCl at 4° (80 g of spores/l.) and centrifuged for 40 min at 6000*g*. The supernatant lacked phospholipase activity and was discarded. The packed spore pellet was placed in a freezing compartment at −20° for at least 12 hr and then transferred to 4° to thaw and germinate over a period of 24–36 hr. Evidence of germination could be seen as a darkening of the spore pellet. Germination was judged to be complete when more than 95% of the spores appeared phase dark in the phase contrast microscope. It was essential that the spores be lyophilized and suspended in 0.1 M NaCl before freezing and thawing to obtain reproducible results.

4. Polyacrylamide gel electrophoresis. For preparative gels, we used the Bistris–Hepes, pH 8.0, buffer system of Jovin (see Chrambach and Rodbard (1971)). The stacking gel was 2.5% acrylamide and 1.25% bisacrylamide, and the resolving gel was 6% acrylamide and 0.16% bisacrylamide. All gels were polymerized by illumination with fluorescent light; riboflavin was present at 0.5 mg/100 ml and *N,N,N′,N′*-tetramethylethylenediamine at 0.05 ml/100 ml. In addition, resolving gel solutions contained ammonium persulfate, 0.075% (w/v), as a polymerization catalyst.

For the analysis of purified phospholipase and an estimate of molecular weight, the sodium dodecyl sulfate system described previously (Scandella and Kornberg, 1971) was used. Gels were 10% acrylamide and 0.275% bisacrylamide. Samples were incubated 5 min at 100° in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol and then dialyzed against upper buffer, 0.1% in sodium dodecyl sulfate, 0.1% in β-mercaptoethanol and 20% in glycerol, before application to gels.

5. Sucrose density gradient sedimentation. The procedure of Martin and Ames (1961) was used to estimate the molecular weight of the spore phospholipase activity and to compare sedimentation properties of the spore and sporangial activities. Samples were loaded onto 3.7-ml 5–20% sucrose gradients. The solutions also contained 10 mM mercaptoethanol, 1 mM EDTA, and 20 mM sodium phosphate, pH 5.5. These were centrifuged at 4° at 55,000 rpm in the SW 56 rotor of the Beckman Model L ultracentrifuge.

6. Phospholipase assay. The assay, similar to that for *Escherichia coli* phospholipase (Scandella and Kornberg, 1971), measures the conversion of chloroform-soluble [^{32}P]PG to water-soluble [^{32}P]lysoPG. The 0.1-ml incubation mixture contained 0.1 M sodium acetate, pH 5.5, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.6 mg/ml of Triton X-100, and 0.3

[1] Abbreviations used are: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; lysoPG, monoacylphosphatidylglycerol; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Hepes, 2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid.

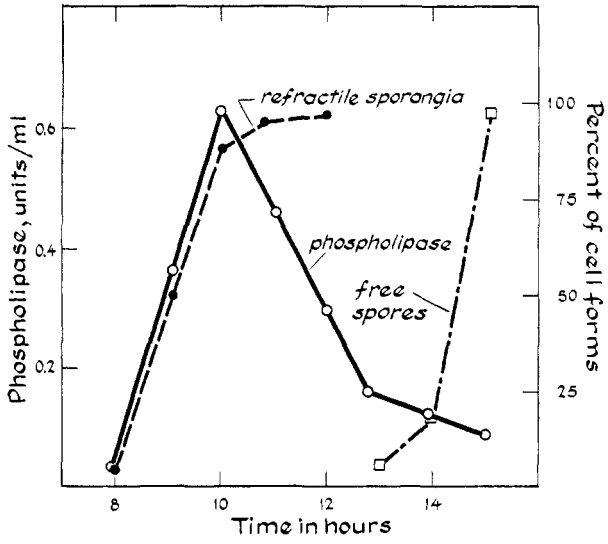

FIGURE 1: Appearance of phospholipase in developing sporangia. The culture was examined at hourly intervals from the start of growth. Refractile forms were estimated by phase contrast microscopy. Sonicated extracts were assayed for phospholipase activity.

mM PG. The radioactive substrate had a specific activity of 0.4–4.0 mCi/mmole. Since the PG was added as 15 μl of a 2 mM stock solution in 95% ethanol, the mixture was 15% in ethanol. The presence or absence of ethanol did not affect the assay. One activity unit is defined as the amount of enzyme that hydrolyzes 1 μmole of substrate per min under assay conditions. For the assay, 20×10^{-6} to 200×10^{-6} units of enzyme were added and the mixture incubated for 30 min at 37°. The reaction was linear over this time period. The substrate levels employed did not permit accumulation of inhibitory levels of fatty acid hydrolysis products. The reaction was halted by the addition of 0.3 ml of chloroform–methanol, 1:2.2 (v/v), to form a single phase. Chloroform (0.1 ml) and water (0.2 ml) were then added to form two dispersed phases which separated on centrifugation for 10 min at 10,000*g*. The aqueous phase contains 85% of the lysoPG but only 1% of the PG. Of the 0.5 ml in the upper water–methanol layer, 0.25 ml was dried on a planchet and counted in a Nuclear-Chicago gas flow counter. This assay procedure would measure other potential water-soluble reaction products, diglyceryl phosphate and glyceryl phosphate. Reaction mixtures, analyzed by tlc in solvent A, contained no radioactively labeled compounds besides PG and lysoPG.

For assessment of phospholipase activity on other substrates, the procedure was modified. Substrate concentrations were 0.1 to 0.3 mM, except for lysoPG, which was 0.04 mM. Reactions were stopped by heating for 2 min at 80°, and the entire reaction mixtures were spotted on tlc plates. For analysis of hydrolysis of PE, lysoPG, diphosphatidylglycerol, lecithin, and glucosaminylphosphatidylglycerol, the plates were developed with solvent A which resolves these substrates and their hydrolysis products. Labeled compounds were located by radioautography, then scraped, and counted. Phosphatidic acid hydrolysis was followed similarly, except that tlc plates impregnated with oxalic acid were used and solvent C was used for development. Tributyrin and its hydrolysis products were separated by tlc with solvent D and the amount of material in the dibutyrin spots estimated by the extent of charring with 30% sulfuric acid spray.

7. PROTEIN ASSAY. Protein was determined by the method of Lowry *et al.* (1951) after precipitation with cold 6% trichloroacetic acid. For the assay of solutions containing Bistris buffer, it was necessary to wash the protein precipitate once with the precipitant.

8. PHOSPHORUS ASSAY. Phosphorus was assayed by the micro method of Chen *et al.* (1956). This assay was used to determine the specific activity of all radioactively labeled phospholipids.

TABLE I: Purification of the Spore Phospholipase.

Fraction	Total Units	Yield (%)	Sp Act. (Units/mg of Protein)
Crude extract	11,400	(100)	9.2
Ammonium sulfate	3,070	27	22.5
DEAE-cellulose	2,700	24	111
Acrylamide gel	1,800	16	357
Hydroxylapatite	600	5.5	1560

Results

A. Sporangial Phospholipase. Phospholipase activity was detected in sporangia, as described in Methods, at about the time when developing spores first become refractile (Figure 1). Subsequently the phospholipase levels decline. These levels do not include enzyme activity in spores inasmuch as stronger sonication would have been required to break open refractile spores contained within the sporangia. Similar results were obtained when sporangia were opened by freezing and thawing, or by treatment with lysozyme or Triton X-100. These procedures also left refractile spores intact. No phospholipase activity (<0.03% of maximal level per spore) was detected in extracts of log phase vegetative cells.

B. Localization of Phospholipase in the Spore. Spores heated for 10 min at 60° remained viable but released no phospholipase upon germination or sonication; sonicates of unheated, ungerminated spores retained their phospholipase activity. Thus heating destroyed the phospholipase in the spore. Phospholipase in sporangial and spore extracts was similarly heat labile as was the purified spore enzyme preparation.

Other spore enzymes, including catalase, adenosine deaminase, alanine racemase (Lawrence, 1957), glucose dehydrogenase (Sadoff *et al.*, 1965), and DNA polymerase (Falaschi *et al.*, 1965) are stable to heat treatment of the spore, although they are heat labile when extracted or in the vegetative cell. The heat lability of phospholipase in the spore suggests that the enzyme may be located in a distinctive and vulnerable region. The relatively selective release of the phospholipase on germination further suggests a rather external location of the enzyme in the spore, perhaps in or near the exosporium.

C. Purification of the Spore Phospholipase. We chose to purify the phospholipase released from germinated spores because the activity per cell was 5 times that of sporangial extracts and the specific activity was 50 times greater. The purification scheme is summarized in Table I.

1. EXTRACTION OF GERMINATED SPORES. Lyophilized spores (76 g) were germinated as described and immediately suspended at 4° in 0.1 M sodium carbonate–sodium bicarbonate buffer, pH 10.9, 5 mM in β-mercaptoethanol to a volume of 590 ml. The suspension was centrifuged for 1 hr at 6000*g* and the

cloudy supernatant was then centrifuged for 1 hr to yield 500 ml of a clear yellowish supernatant (pH 9.6). This extract is unstable and was directly treated with ammonium sulfate.

The yield of enzyme activity was maximal with this extraction at high pH. At lower pH values the yield was reduced, while at higher pH values the activity was destroyed. When germinated spores were extracted above 4° or not extracted immediately the specific activity was lower. Compared to the yield of enzyme in sonicates, about 75% was obtained by the germination procedure (150 units per gram of lyophilized spores).

2. AMMONIUM SULFATE FRACTIONATION. To 500 ml of crude extract was added 72 g of ammonium sulfate, with stirring, at 4°. After 5 min the suspension was centrifuged for 40 min at 6000*g*; 84.5 g of ammonium sulfate was added to the clear yellow supernatant. The precipitate collected by centrifugation was suspended in buffer A (0.018 M Tris-HCl, pH 8.0 at 25°, 1 mM in dithiothreitol) to a volume of 7 ml. This fraction was stable for several months at −20°.

3. DEAE-CELLULOSE CHROMATOGRAPHY. The ammonium sulfate fraction (7 ml) was dialyzed for 3 hr against two successive 1-l. portions of buffer A, 0.05 M in KCl. The dialyzed suspension was centrifuged at low speed; 8.3 ml of clear supernatant was applied to a DEAE-cellulose column (5 $cm^2 \times 20$ cm) previously equilibrated at 4° with buffer A, 0.05 M in KCl. The column was then washed with 25 ml of this buffer. The phospholipase activity was eluted with 800 ml of a linear gradient, 0.05–0.25 M KCl in buffer A. Fractions (12 ml) were collected at a flow rate of 40 ml/hr. Peak fractions were pooled and concentrated to a volume of 9 ml by dialysis against solid polyethylene glycol. This fraction was stable at −20°. Although the yield of the ammonium sulfate fraction varied for different preparations, the overall recovery at the DEAE-cellulose step was about the same.

In some preparations of the phospholipase, the DEAE-cellulose chromatography yielded approximately equal amounts of enzyme activity in each of two peaks. A peak of activity which eluted at a lower salt concentration yielded on rechromatography about 70% of the phospholipase at the usual higher salt concentration. Material from both peaks sedimented on sucrose density gradients at about the same velocity. Varying ionic conditions prior to the DEAE-cellulose column also did not affect the chromatography.

4. PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS. The DEAE-cellulose fraction was dialyzed for 4 hr against two successive 1-l. portions of the electrophoresis upper buffer (20% in glycerol (v/v)). The volume decreased to 5.5 ml. Bromophenol Blue (0.1%, 10 μl) was added as a tracking dye. The sample was electrophoresed at 2° in a preparative polyacrylamide gel apparatus (Buchler Instruments) with the Hepes–Bistris buffer system described in Methods. The lower gel height was 4.0 cm and the upper gel 1.5 cm. The sample was layered onto the upper gel and run at a constant current of 40 mA. Fractions (5.8 ml) were collected every 15 min. Peak fractions were pooled and concentrated to 5.3 ml by dialysis against solid polyethylene glycol.

5. HYDROXYLAPATITE CHROMATOGRAPHY. The polyacrylamide gel fraction (5.1 ml) was dialyzed for 3 hr against two successive 500-ml portions of buffer B (0.01 M potassium phosphate, pH 6.5, 1 mM in dithiothreitol). The dialyzed material was applied to a hydroxylapatite column (0.9 $cm^2 \times$ 19.5 cm) previously equilibrated with buffer B at 4°. The column was washed with 9 ml of buffer B and then the activity was eluted with a 200-ml linear gradient of 0.01 to 0.04 M potassium phosphate, 1 mM in dithiothreitol. Fractions (3 ml) were collected at a flow rate of 2.5 ml/hr. Fractions with high specific activity were pooled and concentrated by dialysis against solid polyethylene glycol. This hydroxylapatite fraction, which will be referred to hereafter as the purified spore preparation, was stable for several months when stored frozen at −20°.

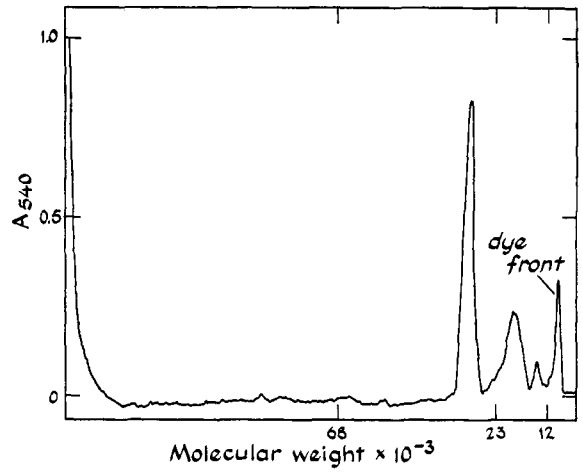

FIGURE 2: Sodium dodecyl sulfate–acrylamide gel electrophoresis of purified spore phospholipase (8 μg). A gel stained with Coomassie Blue was scanned at 547 nm with a Gilford scanner. The stained material at the top of the gel was an artifact of destaining.

D. Electrophoretic Analysis of the Purified Phospholipase. The spore phospholipase preparation was analyzed on an 8% acrylamide gel in the same buffer system as used in the purification of the enzyme. The activity on the gel was located at the same position as the single band of stained protein seen on another gel run in parallel. The activity eluted from the gel and then applied to a sodium dodecyl sulfate acrylamide gel again migrated as a single band to a position corresponding to a molecular weight of 26,000. The purified preparation when applied directly to a sodium dodecyl sulfate gel showed a major band, containing about 75% of the stained material, at a molecular weight position of 26,000 (Figure 2). Judged solely on this basis the spore phospholipase may be 75% pure.

E. Sedimentation Analysis of the Spore and Sporangial Phospholipases. The purified spore phospholipase activity sedimented at 2.7 S in a sucrose gradient velocity experiment (Figure 3), a value which corresponds to a molecular weight of 29,000 for a globular protein. Recovery of enzyme activity in this band was 40%.

Before sedimentation analysis, the crude sporangial extract was first treated with 0.1 volume of 5% streptomycin sulfate (to eliminate nucleic acids and particulate material) and centrifuged for 1 hr at 100,000*g*. The supernatant fraction, which contained 90% of the original activity, showed a broad peak of activity, on a sucrose gradient, centered at 4.5 S with considerable tailing toward the top of the tube; 60% of the activity was recovered. In a parallel experiment, an excess of the purified spore enzyme was added to the sporangial fraction and the mixture analyzed on a sucrose gradient. All the spore activity was shifted to the 4.5 S position; 65% of the activity was recovered. Thus, in crude extracts the phospholipase activity assumes a faster sedimenting form.

F. Optimal pH and Ionic Conditions for PG Hydrolysis. A comparison of the purified spore enzyme and the crude sporangial extract showed the optimal pH to be between 5 and 6.5, depending on the particular buffer used (Figure 4).

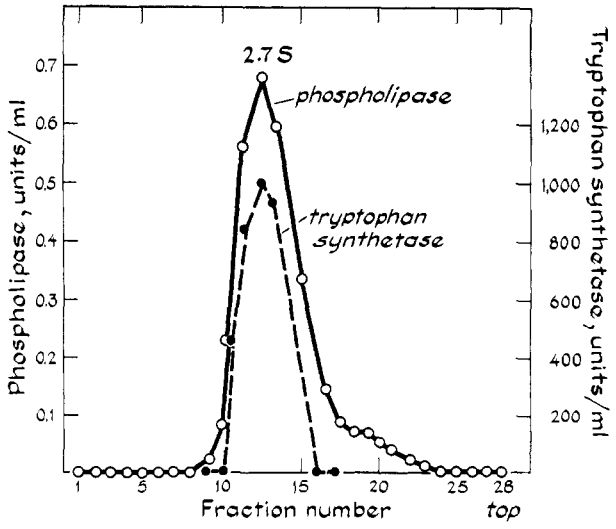

FIGURE 3: Sedimentation velocity of spore phospholipase. Enzyme (0.87 unit) was loaded with a sucrose gradient as described. The sample was centrifuged for 22 hr at 297,000*g*. Tryptophan synthetase A-protein, kindly provided by Dr. K. Kirschner, was used as a sedimentation marker ($s_{20,w}$ = 2.7; molecular wt = 29,000). Recoveries of phospholipase and synthetase were 40% and 70%, respectively.

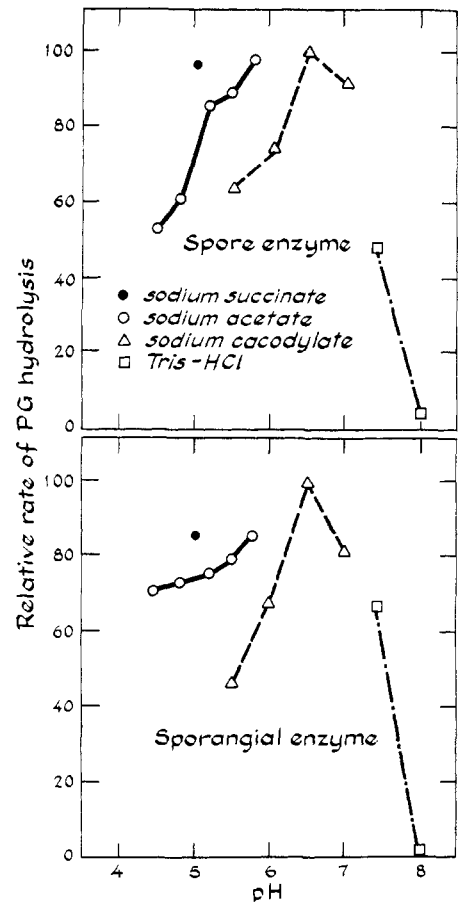

FIGURE 4: Hydrolysis of PG by spore and sporangial phospholipases (3.8×10^{-5} and 11.1×10^{-5} unit, respectively) as a function of pH. Buffer concentrations were 0.1 M.

At an ionic strength of 0.2 the hydrolysis rate was reduced by 50% and higher salt concentrations inhibited activity still further. Phospholipase activity was not affected by Ca^{2+} (1 mM), Mg^{2+} (1 mM), or EDTA (1 mM). Other phospholipases (A_1) which do not require Ca^{2+} have been reported in calf brain (Gatt, 1968), rat liver microsomes (Waite and Van Deenen, 1967), and rat liver lysosomes (Franson *et al.*, 1971).

G. Effect of Detergents on PG Hydrolysis. The action of the purified spore phospholipase required the presence of detergents. Either nonionic Triton X-100 or anionic sodium taurocholate stimulated PG hydrolysis about 25-fold (Figure 5). When both detergents were present together there was no further enhancement in rate. At pH 7, sodium deoxycholate had an effect similar to that of taurocholate.

Sodium dodecyl sulfate, another anionic agent, inhibited hydrolysis 2-fold at 0.01% and over 100-fold at 0.1%.

The requirement for detergent by the crude sporangial phospholipase was similar but less marked (Figure 5); the stimulation was only about twofold.

H. Specificity of the Phospholipases. 1. POSITIONAL SPECIFICITY. Specificity of cleavage at the 1-acyl linkage was demonstrated with the synthetic substrate, *rac*-1-oleoyl-2-palmitoylglycero-3-phosphorylglycerol. Purified spore enzyme (0.043 unit) was incubated with substrate (36 nmoles) under standard assay conditions. The lysoPG product, purified by thin-layer chromatography in solvent A, was subjected to methanolysis and gas–liquid chromatographic analysis of the resultant fatty acid methyl esters. Palmitate (13 nmoles, uncorrected for losses on methanolysis) constituted more than 95% of the fatty acid in the lysoPG. Similar results were obtained when the phospholipase A_1 specificity of the crude sporangial activity was tested.

2. ACTION ON TRIGLYCERIDES. To examine the purified spore enzyme for lipase activity, tributyrin (1.4 μmoles) served as a substrate with sodium taurocholate (10 mg/ml) present. About 1 nmole of the product, dibutyrin, was detected by thin-layer chromatography after incubation with 0.04 unit of enzyme. This rate of hydrolysis of tributyrin was only 0.2% that of L-lecithin, under the same conditions. The possibility remains that the low level of triglyceride hydrolysis may be due to impurities in the enzyme preparation rather than to lack of specificity of the purified spore phospholipase.

3. PHOSPHOLIPID HEAD GROUP SPECIFICITY. PG, the major phospholipid of this *B. megaterium* strain, was by far the best substrate for the purified spore enzyme when tested with Triton (Table II). However PE, which was virtually inert under these conditions, was almost as good a substrate in the presence of an anionic detergent. The 100-fold increase in rate of PE (and phosphatidylcholine) hydrolysis induced by sodium taurocholate suggests that the enzyme discriminates between phospholipids on the basis of charge. The stimulatory effect of the anionic detergent on the hydrolysis of neutral phospholipids is consistent with a preference of the enzyme for a substrate or substrate–detergent micelle that is negatively charged. LysoPG was tested at a much lower concentration; further study with varied conditions and concentrations might show it to be more effective as a substrate. [^{14}C]Lecithin used in these experiments contained 85% of its [^{14}C]palmitoyl groups in the 2-acyl position, as shown by analysis with *Crotalus adamanteus* venom (phospholipase A_2). After hydrolysis with *B. megaterium* phospholipase, 15% of radioactive product was fatty acid and 85% was lysolecithin.

The behavior of the sporangial phospholipase on PG and PE showed the same relative rates and dependence on detergents as did the spore phospholipase

I. Kinetics of PG Hydrolysis by the Spore Phospholipase. For hydrolysis of PG by the spore enzyme, $K_{m,app} = 6 \times$

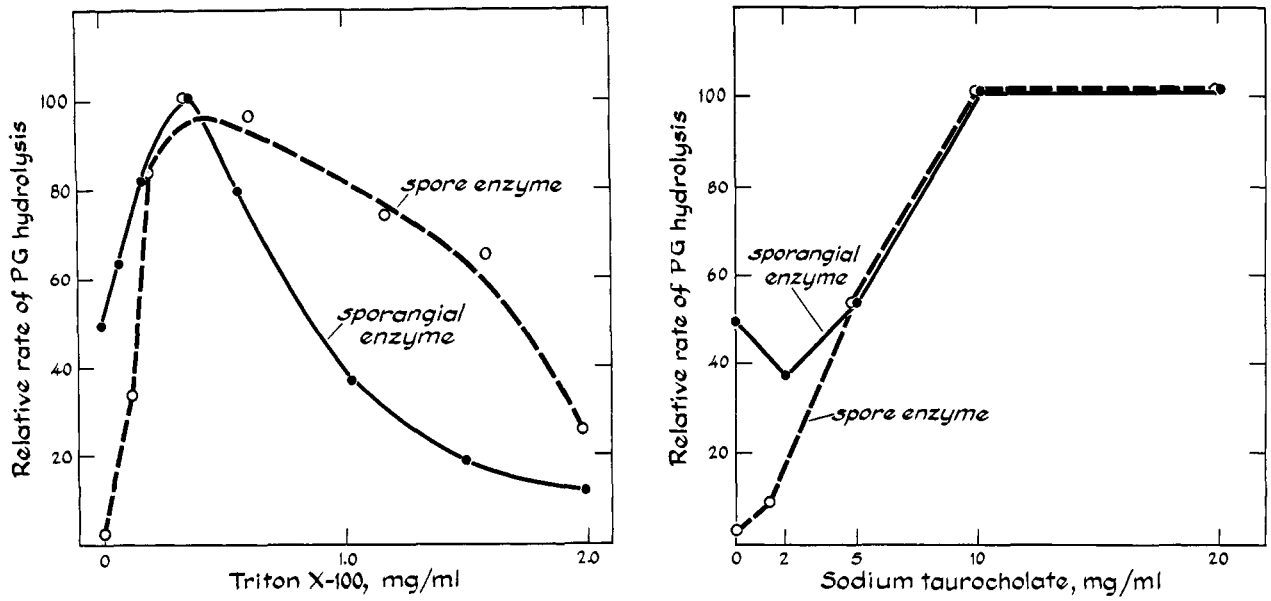

FIGURE 5: Influence of detergents on the hydrolysis of PG by spore and sporangial phospholipases (5.4×10^{-5} and 9.7×10^{-5} unit, respectively). Reaction conditions were as in the standard assay, except that detergent concentration was varied, and sodium taurocholate replaced Triton X-100 where indicated.

10^{-5} M was estimated from a Lineweaver–Burk plot; the turnover number, based on the molecular weight of 26,000 was 8400 moles/mole per min. Spore enzyme (0.3 ng) was incubated with varying PG concentrations under standard conditions, except that volumes were doubled for convenience. Fatty acid inhibited the reaction; palmitate at 5×10^{-4} M lowered the rate by 50%

J. Phospholipase Activity on a Membrane Preparation. The spore phospholipase hydrolyzed *B. megaterium* and *E. coli* PG at the same rate indicating no preference by the enzyme for the rather distinctive fatty acyl chains of the phospholipids of these two organisms. The capacity of the enzyme to attack a plasma membrane was tested with a ^{32}P-labeled *E. coli* preparation (Schnaitman, 1970a,b). On the basis of conversion of phospholipid to a water-soluble form, with and without Triton X-100 present, hydrolysis was 100 times slower than that of pure PG under optimal conditions. Thin-layer chromatography of an entire membrane digest indicated that PG and PE were hydrolyzed at comparable rates under our standard assay conditions. The rate of attack on membranes was similar to that on a pure phospholipid in the absence of an appropriate detergent. Conceivably a lack of loci of net negative charge on the membrane surface may account for the relative failure of the enzyme to attack it.

Discussion

Similar physical and enzymatic properties suggest that the phospholipase activities observed in sporangia and mature spores are identical. The enzymes from both sources are heat labile and show similar sedimentation behavior. The activities have common pH optima, are stimulated by the same detergent concentrations, have no metal requirements, and show similar specificity for substrates. Optimal detergent concentrations stimulated the two activities to different extents; this can be accounted for by the effects of crude extracts, as discussed below. It is plausible that an enzyme activity which appears in the sporangial cytoplasm during sporulation would also be found in the spore.

Phospholipase first appears in sporangia at the time when an extensive decline in the total phospholipid level begins (Bertsch *et al.*, 1969), well before lysis of the sporangium. The amount of sporangial enzyme, as judged by release upon light sonication, increases and then decreases. Phospholipase activity was not detected in the culture medium. The decline in sporangial activity might be due to degradation of the enzyme, or, in part to its inclusion in outer layers of the developing spore coat. Although spores are refractile when the phospholipase appears, they are not fully mature, as indicated, for example, by the fact that heat resistance is acquired only later in spore development (Kornberg *et al.*, 1968).

We estimate that a mature spore contains about 3000

TABLE II: Influence of Phospholipid Head Group on Phospholipase Action.

Substrate	Rel Hydrolysis Rate[a]	
	Triton X-100 (0.6 mg/ml)	Sodium Taurocholate (10 mg/ml)
Negative charge		
Phosphatidylglycerol	100	100
Diphosphatidylglycerol	2.0	2.0
Phosphatidic acid	1.4	4.5
1-Acyllysophosphatidylglycerol	0.61	0.61
Neutral charge		
Phosphatidylethanolamine	0.26	49
Phosphatidylcholine	0.21	29
Glucosaminylphosphatidylglycerol	0.37	8.3

[a] Hydrolysis rates are expressed as amount of substrate digested per unit of enzyme added, relative to the rate of phosphatidylglycerol hydrolysis in the presence of Triton.

molecules of phospholipase. Since the spore enzyme can be denatured without affecting spore viability, it cannot be essential to germination. However there are multiple pathways for germination and only some of them may require the phospholipase. The enzyme may be released, as spore proteases are, to function outside the germinated spore. In this way exogenous substrates may be utilized as building blocks and fuels for the nutrition of the germinating spore and developing cell.

The purified spore phospholipase is stimulated by detergents in a striking way. Hydrolysis of the neutral substrate, PE, is enhanced 200-fold by sodium taurocholate, an anion, but not at all by the nonionic Triton X-100. Hydrolysis of other neutral substrates is affected similarly. In the presence of optimal concentrations of either Triton or taurocholate, negatively charged PG is digested 25 times more rapidly. These detergents have equal effects on hydrolysis of other negative phospholipids. Thus, the phospholipase works best on detergent–phospholipid complexes which carry a net negative charge.

Inasmuch as this phospholipase is a water-soluble enzyme and requires a detergent for its action, we wondered what compound was provided in the cell to enable the enzyme to degrade phospholipids there. It seemed significant to us that the phospholipase activity of crude sporangial extracts was already at half of the optimal level attainable by addition of detergents (Figure 5). In a series of preliminary experiments, we have found that a factor is present in boiled extracts of sporangia which takes the place of a detergent in stimulating PG hydrolysis. The factor stimulates the activity of sporangial extracts and the purified spore enzyme to about half of the optimal level. The action of such a heat-stable factor can also be demonstrated in extracts of *B. megaterium* spores and vegetative cells, and of *E. coli*. The factor resembles Triton X-100 rather than sodium taurocholate in that it enhances PG hydrolysis, but not PE hydrolysis. The chemical nature of this factor and how it facilitates the physiological action of the phospholipase now require further study.

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