Synthesis of a thiophosphate analog of dioctanoylphosphatidylcholine: a phospholipase C substrate

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Abstract Dioctanoylthiophosphatidylcholine, a racemic thiophosphate analog of L-α-dioctanoylphosphatidylcholine, has been synthesized and isolated by flash chromatography. In contrast with the didecanoylthiophosphatidylcholine synthesized previously, the analog is easily dispersed on sonication in aqueous media and is rapidly hydrolyzed to produce a free thiol group in the presence of the extracellular phospholipase C from either *Bacillus cereus* or *Clostridium perfringens*. When 5,5'-dithiobis (2-nitrobenzoic acid) was included as a thiol reactive chromogenic agent, the resultant measurement of product release, as an increase in absorbance at 412 nm, showed a linear relationship with added enzyme.—**Snyder, W.R.** Synthesis of a thiophosphate analog of dioctanoylphosphatidylcholine: a phospholipase C substrate. *J. Lipid Res.* 1987. **28:** 949–954.

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Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) catalyzes the hydrolysis of the glycerophosphate bond in membrane phospholipids. The hydrolysis of phosphatidylcholine, for example, results in the production of a diglyceride and a water-soluble choline phosphomonoester. The resultant alteration of the amphipathic nature of the substrate phospholipid must result in a significant change in the nature of biological membranes.

In order to explore fully the reaction catalyzed by phospholipase C, it is advantageous to utilize an assay method that continuously measures product formation and also is highly specific for the hydrolysis of the glycerophosphate bond. To date, only the thiophosphate analog assay (1) satisfies both of these criteria.

Cox, Snyder, and Horrocks (1) synthesized phospholipid analogs where the glycerophosphate oxygen was replaced by a sulfur atom. After phospholipase C-catalyzed hydrolysis, a thiol-containing product (thiodiglyceride) was formed. Under mild aqueous conditions, this thiol could be reacted with a chromogenic agent to measure the continuous formation of product. The synthetic phosphatidylcholine analog substrates contained either one 16-carbon acyl chain (a mercaptoethanol derivative) or two 10-carbon acyl chains (a mercaptoglycerol derivative) (diC_{10}-S-PC). Although the latter analog has the advantage of being structurally more similar to the natural substrate for phospholipase C, it proved to be difficult to disperse and resulted in relatively slow hydrolytic rates.

A readily dispersible, rapidly hydrolyzed thiophosphate analog, dioctanoylthiophosphatidylcholine (diC_8-S-PC) has been synthesized and used to assay the extracellular C-specific phospholipases from *Bacillus cereus* and *Clostridium perfringens*. The organic synthesis, isolation by flash chromatography, and use as a phospholipase C substrate are reported here.

MATERIALS AND METHODS

Octanoylchloride, 3-mercapto-1,2-proapanediol, dithiothreitol (DTT), 2-bromoethanol, anhydrous trimethylamine (TMA), and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Aldrich Chemical Company. Phosphorous oxychloride (Baker) was distilled immediately before use (105–106°C). Triethylamine (TEA) (Eastman) was dried over Linde type 4A molecular sieve, refluxed with KOH pellets, and distilled (88–90°C). Pyridine (Aldrich Gold Label) was dried over Linde type 4A molecular sieve before use. Chloroform (Baker) was extracted with two volumes of water to remove the ethanol stabilizer, dried over $CaCl_2$, refluxed with $CaSO_4$, and distilled. MOPS, 3-(N-morpholino)propanesulfonic acid,

Abbreviations: diC_8-S-PC, dioctanoylthiophosphatidylcholine (*rac*-3-S-phosphocholine-1,2-O-dictanoyl-3-mercapto-1,2-propanediol); diC_{10}-S-PC, didecanoylthiophosphatidylcholine; diC_8PC, L-α-dioctanoylphosphatidylcholine; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); MOPS, 3-(N-morpholino)propanesulfonic acid.

L-α-dioctanoylphosphatidylcholine (diC_8PC), phospholipase C (*Bacillus cereus*, 3,000 Units / mg of protein), and phospholipase C (*Clostridium perfringens*, 300 Units / mg of protein) were obtained from Sigma Chemical Company and used without further purification. The activity units quoted for the phospholipases are based on the supplier's specifications and were not obtained during this study.

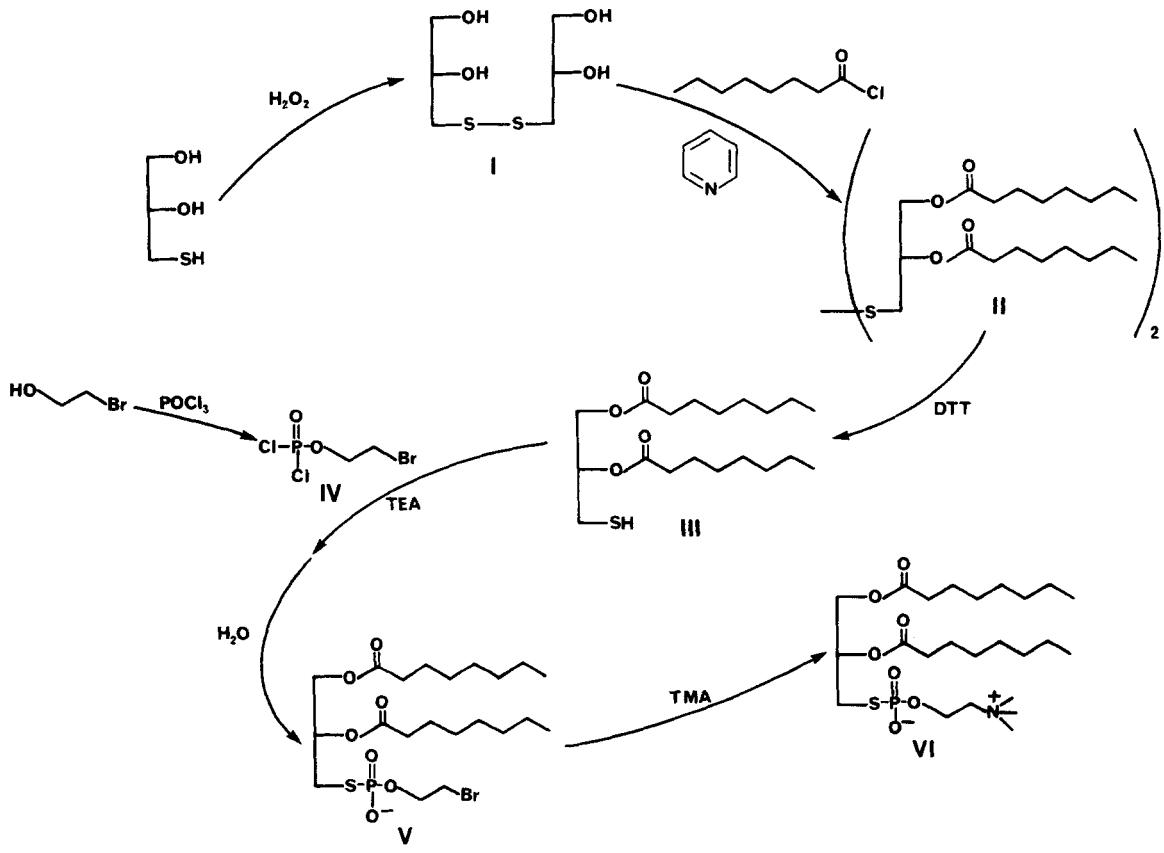
Elemental analyses (C,H,N) were performed on a Perkin Elmer 240 Elemental Analyzer. Infrared spectra were recorded on a Mattson Cygnus 25 FT-IR, and NMR spectra (^1H and ^{31}P) were obtained from an IBM WP-200SY 200 MHz FT-NMR using tetramethylsilane and 85% H_3PO_4 as the respective standards. Thin-layer chromatography was carried out on glass-backed silica gel 60 plates (E. Merck) and material was generally detected by exposure to I_2 vapor. Phosphate-containing compounds were stained with a molybdate spray (2), thiols were detected with a DTNB spray (1), and choline-containing materials were identified with Dragendorff reagent (2).

Preparation of diC_8-S-PC

The dioctanoyl phosphatidylcholine thiophosphate analog (VI) (**Scheme 1**) was prepared as a racemic mixture by a modification of the procedure of Cox et al. (1).

Compound I, *rac*-3,3'-dithiobis (1,2-propanediol) was prepared by a variation of the method of Danehy and Hunter (3). Eighty grams (0.74 mol) of 3-mercapto-1,2-propanediol was placed in a flask and cooled in an ice bath. Forty two ml of 30% hydrogen peroxide was added dropwise to the rapidly stirring thiol while the temperature was maintained between 30° and 40°C. When the exothermic reaction was complete, the mixture was stirred overnight at room temperature. Water was removed from the product in a rotary evaporator (40°C) with successive additions of absolute ethanol. The dried product was a chalky white solid which was used without further purification. Anal. calcd. for $C_6H_{14}S_2O_4$: C, 33.63; H, 6.58. Found: C, 33.60; H, 6.54.

The preparation of *rac*-3,3'-dithiobis (1,2-O-dioctanoyl-1,2-propanediol) (compound II) was carried out by a modification of the procedure used by Cox et al. (1) for the synthesis of diC_{10}-S-PC. Thirty mmol of the disulfide (I) was dissolved in 200 ml of ethanol-free chloroform and 180 mmol of dry pyridine. Octanoylchloride (150 mmol) was added with rapid stirring, and the mixture was stirred overnight at room temperature. The workup of the mixture and purification by basic alumina chromatography



Scheme 1. Synthetic scheme.

have been reported (1). FT-IR(thin film): 2950, 2920, 2850, 1460, 1375 cm^{-1}(C-H); 1740, 1150 cm^{-1}(ester); 1105 cm^{-1}(secondary ester); 1055 cm^{-1}(primary ester). ^{1}H-NMR ($CDCl_3$); δ(ppm): 0.9, [6H, apparent triplet, $(CH_2)nC\underline{H}_3$]; 1.3, [16H, apparent singlet, $-(C\underline{H}_2)n-CH_3$]; 1.6, [4H, multiplet, $-C(O)-CH_2-C\underline{H}_2-$]; 2.3, [4H, multiplet, $C(O)-C\underline{H}_2-$]; 2.9, [2H, doublet, $-C\underline{H}_2-S-$]; 4.0-4.5, [2H, complex, $-C\underline{H}_2-O-C(O)-$], 5.3, [1H, multiplet, $\underline{H}-C-O-$].

Compound III (*rac*-3-mercapto-1,2-O-dioctanoyl-1,2-propanediol) was prepared by dissolving 15 mmol of compound II in 300 ml of absolute ethanol and stirring with 30 mmol of dithiothreitol and 0.6 ml of 29% aqueous ammonia for 15 min at room temperature. The ethanol was removed by rotary evaporation (40°C) and the free thiol (III) was isolated as previously described (1). The infrared spectrum of compound III was identical to that reported for II except for the appearance of a band at 2570 cm^{-1} (S-H).

Bromoethylphosphoric acid dichloride (IV) was prepared by the method of Eibl and Nicksch (4) except that the final product was distilled (89°C/1.5 mm Hg) and stored in sealed ampules at -20°C. ^{1}H-NMR; δ(ppm): 3.65, [2H, multiplet, $-C\underline{H}_2-Br$]; 4.6, [2H, multiplet, $-C\underline{H}_2-O-P-$]. (The methylene protons are coupled to each other with a J = 5.8 Hz. The higher field "triplet" at δ = 3.65 ppm is coupled to P with a $^4J_{PH}$ = 1.2 Hz, while the lower field "triplet" at δ = 4.6 ppm is coupled to P with a $^3J_{PH}$ = 9.6 Hz.)

The synthesis of compound V and subsequent trimethylation to produce compound VI were performed as described by Cox et al. (1) and Aarsman, van Deenen, and van den Bosch (5). Following the evaporation of the solvent, the crude compound VI (20 mmolar scale) was dried in vacuo, dissolved in a minimal volume (40 ml) of chloroform-methanol 1:4 and applied to a dry packed (30 cm × 6.0 cm) silica gel 60 column (E. Merck for preparative thin-layer chromatography). The column used was similar to that described by Still, Kahn, and Mitra (6) except that it contained a coarse fritted disk for packing support. The material was eluted with chloroform-methanol 1:4 under a moderate N_2(g) pressure of 11 psi. A flow rate of 10-12 ml/min was maintained and 20-24 ml fractions were collected. The eluted fractions were monitored by thin-layer chromatography in chloroform-methanol-water 60:40:10. Fractions containing diC_8-S-PC were pooled and used for subsequent studies. The overall yields for the conversion of III to VI were typically between 30 and 40%. Anal. calcd. for $C_{24}H_{48}PNSO_7H_2O$: C, 53.02; H, 9.27; N, 2.58. Found: C, 53.08; H, 9.35; N, 2.53. FT-IR(thin film): 3368 cm^{-1} (bound water); 2955, 2930, 2857, 1470, 1387 cm^{-1} (C-H); 1740, 1165 cm^{-1} (ester); 1254 cm^{-1} (symmetric PO_2^-); 1076 cm^{-1} (asymmetric PO_2^-); 1055 cm^{-1}((P)OC); 968, 926 cm^{-1} (trimethylammonium); 875 cm^{-1} (PO(C)). ^{1}H-NMR ($CDCl_3$); δ (ppm): 0.9, [6H, apparent triplet, $(CH_2)_nC\underline{H}_3$]; 1.3, [16H, apparent singlet, $-(C\underline{H}_2)_4-CH_3$]; 1.6, [4H, multiplet, $-C(O)-CH_2-C\underline{H}_2-$]; 2.3, [4H, multiplet, $C(O)-C\underline{H}_2-$]; 3.0, [2H, multiplet, $-C\underline{H}_2-S-P-$]; 3.4, [9H, singlet, $-N(-C\underline{H}_3)_3$]; 3.5 - 5.2, [7H, complex, $-C\underline{H}_2-N$, $-C\underline{H}_2-O-C(O)-$, $-P-O-C\underline{H}_2-$, $\underline{H}-C-O-$]. ^{31}P-NMR ($CDCl_3$); δ = 15.7 ppm.

Enzyme assay

An aliquot of diC_8-S-PC in chloroform was placed in a glass vial, and the solvent was removed under a stream of N_2(g). The substrate was dispersed on a vortex mixer at room temperature in 0.05 M MOPS buffer (pH 7.3) at a final concentration of 2.5 mM diC_8-S-PC. The stock solution was sonicated (150 watts) for 30 sec with a Branson Sonifier cell disruptor Model 350A equipped with a 0.5-inch disruptor horn.

Into a 1.2-ml optical glass cuvette were added 200 μl of the diC_8-S-PC stock solution, 200 μl of 5.0 mM DTNB in 0.05 M MOPS buffer (pH 7.3), and sufficient volume of additional 0.05 M MOPS buffer (pH 7.3), to result in a final assay volume of 1.0 ml after enzyme addition. When the phospholipase C from *Clostridium perfringens* was utilized, the assay mixture included 25 mM $CaCl_2$. The cuvette was placed in the thermostated (37°C) sample compartment of an LKB Ultrospec 4050 UV-Vis Spectrophotometer. After temperature equilibration and observation of a stable baseline absorbance, an aliquot of phospholipase C was added. The resultant absorbance change at 412 nm was recorded, and the rate of hydrolysis was determined using a molar absorbtivity of 12,800 $M^{-1}cm^{-1}$ (5,7) for the 2-nitro-5-thiobenzoate dianion produced.

RESULTS AND DISCUSSION

The thiophosphate derivative of dioctanoylphosphatidylcholine, diC_8-S-PC, has been synthesized by a modification of the method reported by Cox et al. (1). The use of H_2O_2 for the oxidation of 3-mercapto-1,2-propanediol has proven to be an excellent method by which large quantities of the disulfide (compound I) can be prepared. The essentially odorless solid has been stored (desiccated at room temperature) for at least 6 years without apparent degradation. The procedure of Eibl and Nicksch (4) for the preparation of bromoethylphosphoric acid dichloride has led to improved yields compared with earlier methods that involved heating to drive off the HCl(g) produced during the synthesis. Current investigation is underway to improve the phosphorylation reaction which presently leads to the formation of considerable by-products when thiol reactants are used.

The difficulties associated with product isolation due to the low yield thiol phosphorylation step have been over-

come by the utilization of a silica gel flash column chromatography system. **Fig. 1** shows thin-layer chromatograms obtained by spotting fractions eluted from the silica gel column. At the origin (labled L), a sample of the trimethylation product (compound VI) was spotted after drying and dissolving in chloroform–methanol 1:4. This multiple component mixture was applied to the silica column. The remaining numbered lanes (5–185) were obtained from the eluted fractions (chloroform–methanol 1:4) that were subsequently collected. While the early fractions contained substantial contaminants, fractions 75–140 contained essentially pure diC_8-S-PC. The same column technique has been used to purify several different thiophosphorylcholine analogs with equally successful results.

The IR spectrum for diC_8-S-PC was virtually identical to that obtained with a similar thin film of diC_8PC. The only significant difference was an extra signal at 824 cm^{-1}, which was present with diC_8PC, but absent in the spectrum of the thiophosphate analog (diC_8-S-PC). The observation is analogous to that observed by Cox et al. (1), and the band has been attributed to an -O-P-O- antisymmetric stretch (8), which should be absent in the thiophosphate analog.

A single difference was also observed when the ^1H NMR spectra of diC_8PC and diC_8-S-PC were compared. A two-proton multiplet at δ = 4.0 ppm was observed in the spectrum of diC_8PC which can be assigned to the $-C\underline{H}_2-O-P$ protons (9). This signal was absent in the ^1H NMR spectrum of the thiophosphate analog (diC_8-S-PC) and was replaced by a two-proton multiplet at δ = 3.0 ppm, which is consistent with the replacement of oxygen with sulfur ($-C\underline{H}_2-S-P$). In the ^{31}P NMR, diC_8PC appeared slightly upfield from the reference (85% H_3PO_4) at δ = -0.21 ppm, while the signal obtained for the thiophosphate (diC_8-S-PC) was shifted downfield to δ = 15.7 ppm. The magnitude of this chemical shift is consistent with that observed for other thiophosphate analogs (10, 11).

The products resulting from phospholipase C-catalyzed hydrolysis of diC_8-S-PC have been identified by thin-layer chromatography on silica gel 60 plates. After incubation with the *Bacillus cereus* enzyme, a product extracted with chloroform–methanol 2:1 was shown to cochromatograph (chloroform–diethylether 9:1) with a sample of compound III, both staining positively with DTNB spray reagent (1). The water-soluble reaction product cochromatographed (butanol–acetic acid–water 5:3:1) with a sample of phosphocholine, and stained with molybdate spray (2) as well as Dragendorff reagent (2). The hydrolysis of diC_8-S-PC catalyzed by phospholipase D was shown to produce a relatively unstable thiophosphate analog of phosphatidic acid which is currently under investigation (Nyquist, D.A., and Snyder, W.R., unpublished results).

The use of diC_8-S-PC as a phospholipase C substrate has proven to be a significant improvement over the diC_{10}-S-PC analog. The diC_8-S-PC substrate is easily dispersed with a 30-sec sonication to provide a clear suspension, which is hydrolyzed in the presence of the C-specific phospholipases at rates that are at least five times greater than those observed with diC_{10}-S-PC. **Fig. 2** shows representative time-course data for the assay of the phospholipases C from *Bacillus cereus* (Fig. 2A) and *Clostridium perfringens* (Fig. 2B) using diC_8-S-PC in the presence of DTNB. After essentially no change in absorbance at 412 nm with substrate and DTNB alone, the resulting changes in the absorbance following the addition of the enzymes are indicated by the arrows. Under these assay conditions (pH 7.3, 37°C), both of the enzymes show an initial lag phase which is more pronounced with the enzyme from *Clostridum perfringens* (Fig. 2B). While conditions can be varied to eliminate the lag phase with

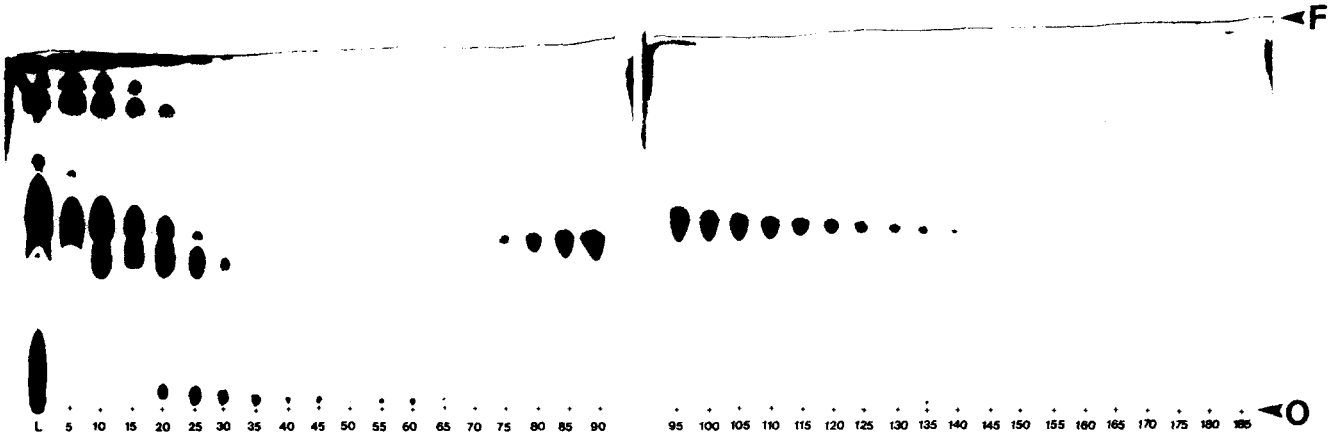


Fig. 1. TLC of the fractions obtained from flash silica chromatography used to purify the crude synthetic diC_8-S-PC (L). O indicates the origins of sample application and F, the solvent front. The numbers indicate the fractions collected. Chloroform–methanol–water 6:4:1 (by vol) was used as the developing solvent, and the material was visualized by exposure to I_2 vapor.

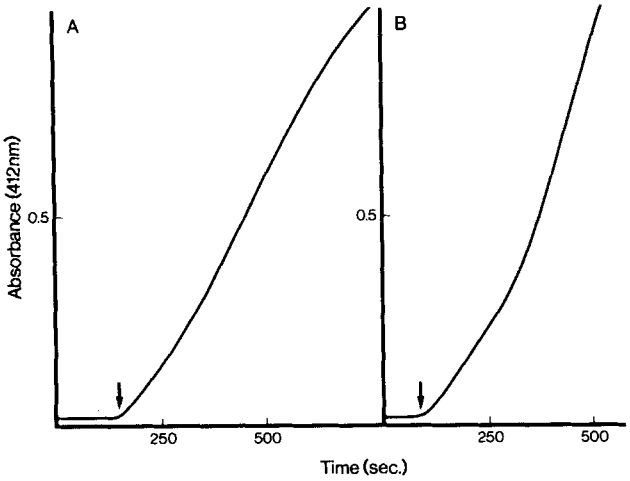


Fig. 2. Time courses for the hydrolysis of diC_8-S-PC by phospholipase C in the presence of DTNB in a total assay volume of 1 ml. A. Incubation mixture contained 200 μM diC_8-S-PC and 1.0 mM DTNB in 0.05 M MOPS buffer, pH 7.3. The arrow indicates the addition of 0.5 Units (Sigma Chemical) of the *Bacillus cereus* phospholipase C. B. Incubation mixture contained 200 μM diC_8-S-PC, 1.0 mM DTNB, and 25 mM Ca^{2+} in 0.05 M MOPS buffer, pH 7.3. The arrow indicates the addition of 0.5 Units (Sigma Chemical) of the *Clostridium perfringens* phospholipase C. Both reactions were carried out at 37°C.

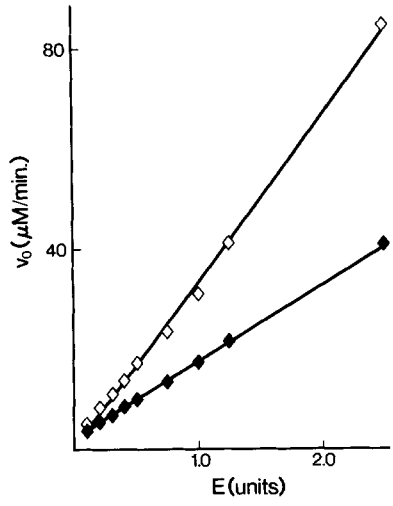


Fig. 3. Initial velocities as a function of enzyme concentration. One-ml assay volumes (37°C) contained 250 μM diC_8-S-PC and 1.0 mM DTNB in 0.05 M MOPS buffer, pH 7.3 (25 mM Ca^{2+} was included for the *Clostridium perfringens* enzyme only). The units of enzyme added (◇, *Clostridium perfringens* and ◆, *Bacillus cereus*) are based on the supplier's (Sigma) specifications.

the *Bacillus cereus* enzyme (Snyder, W.R., unpublished results), the lag is observed under all tested experimental conditions for the *Clostridium perfringens* enzyme including preincubation of the enzyme with Ca^{2+} (Riedy, G. P., and W. R. Snyder, unpublished results).

The initial rate for each of the phospholipase C-catalyzed reactions is defined as the maximal velocity attained following the lag period and prior to the eventual rate decrease resulting from substrate depletion. **Fig. 3** demonstrates these measured initial velocities as a function of phospholipase C concentration for the two extracellular bacterial enzymes. Both show a linear relationship between initial velocity and the amount of enzyme added, indicating that the thiophosphate analog spectrophotometric assay is a viable measure of phospholipase C activity for the enzymes from *Bacillus cereus* and *Clostridium perfringens*.

Relative hydrolytic rates were determined for diC_8PC and diC_8-S-PC using the traditional pH stat method (pH 8.0) originally described by Dennis (12) for the assay of phospholipase A_2. The results obtained with the *Bacillus cereus* enzyme showed that the diC_8PC substrate was hydrolyzed approximately 200 times faster than the thiophosphate analog under identical pH stat assay conditions. The rate obtained for the pH stat assay using diC_8PC was 100 times faster than the rate observed for diC_8-S-PC hydrolysis in the spectrophotometric assay. The twofold discrepancy appears to involve a stimulation of the enzyme-catalyzed reaction by DTNB and has been observed with both substrates and assay methods.

The loss in absolute hydrolytic rate observed when the thiophosphate analog is compared with diC_8PC hydrolysis appears to be offset by the gain in sensitivity resulting from the DTNB thiol reaction when diC_8-S-PC is used in the spectrophotometric assay. This coupled with the high degree of assay specificity, and the convenience afforded by a spectrophotometric method makes the technique a viable assay for phospholipase C. Studies are currently in progress to optimize the assay conditions, to investigate the involvement of DTNB in the reaction, and to explore the use of the method for the assay of other C-specific phospholipases.

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