A Phospholipase C from the Dallas 1E Strain of *Legionella pneumophila* Serogroup 5: Purification and Characterization of Conditions for Optimal Activity with an Artificial Substrate

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(Received 8 October 1987)

Phospholipase C from the Dallas 1E strain of *Legionella pneumophila* serogroup 5 was purified from buffered yeast extract culture supernate by ion-exchange chromatography followed by fractionation by manganous chloride and ammonium sulphate precipitation steps. Enzyme activity was assayed by hydrolysis of *p*-nitrophenylphosphorylcholine and confirmed by release of radioactivity from tritiated L-α-dipalmitoylphosphatidylcholine labelled in the methyl groups of choline. After SDS-PAGE, the purified preparation yielded a single band upon Coomassie-blue staining. This protein migrated with an apparent $M_r$ of 50000-54000. Phospholipase C activity was maximal at pH $\geq$ 8.4 and was enhanced in the presence of sorbitol and of several nonionic detergents but was eliminated by SDS. EDTA, Cu$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$ inhibited enzyme activity, whereas Ba$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ restored activity to EDTA-treated material. No haemolytic activity was demonstrated with the purified enzyme.

**INTRODUCTION**

Phospholipase C (lecithinase, EC 3.1.4.3) hydrolyses phosphatidylcholine (lecithin) to 1,2-diacylglycerol and phosphorylcholine. This enzyme is recognized to be a virulence factor of several bacterial pathogens, including *Clostridium perfringens* (Mollby, 1978) and *Pseudomonas aeruginosa* (Berka & Vasil, 1982).

Certain species of *Legionella* also produce phospholipase C, and several potential roles exist for this enzyme in the pathogenesis of legionellosis (Baine *et al*., 1979; Baine, 1985). Hydrolysis of phosphatidylcholine, which is a major component of alveolar surfactant (Klaus *et al*., 1961), could impair pulmonary gas exchange. Phosphatidylcholine is also an important constituent of eukaryotic membranes (Alberts *et al*., 1983). The cytolytic action of phospholipase C from *C. perfringens* and other bacteria is well established (Mollby, 1978), and *Legionella* phospholipase C might injure both lung tissue and inflammatory cells in the host. Lesser alterations of host-cell membranes by phospholipase C may enhance bacterial adherence to target cells (Malmqvist *et al*., 1984). Within phagocytes perturbation of phagosome membranes by hydrolysis of phospholipid might contribute to the failure of phagosome–lysosome fusion that is observed in mononuclear cells ingesting live *L. pneumophila* (Horwitz, 1983) and that presumably permits unimpeded multiplication of the bacteria within the phagocyte.

Phospholipase C from *Legionella* might also affect the host by liberating active reaction products. For example, phospholipase C from *C. perfringens* induces ornithine decarboxylase in lymphocytes, apparently through the mediation of diacylglycerol (Otani *et al*., 1984). Since diacylglycerol activates protein kinase C, an enzyme with broad regulatory functions (Nishizuka, 1986; Waite, 1985), release of diacylglycerol by enzymic degradation of phospholipid could have multiple pathophysiological consequences.

**Abbreviation**: NPPC, *p*-nitrophenylphosphorylcholine.
The Dallas IE strain, isolated in 1978 from a cooling tower in Dallas, Texas, is the type strain for serogroup 5 of *L. pneumophila* (England et al., 1980), although recent taxonomic studies indicate that Dallas IE may more appropriately be regarded as belonging to a separate species of *Legionella* (Selander et al., 1985). Phospholipase C activity has been demonstrated in intact cells of Dallas IE and other strains (Baine, 1985), but purification of this enzyme from *Legionella* has not previously been reported. Dallas IE is more haemolytic than the type strain of *L. pneumophila*, Philadelphia 1, when grown on agar that incorporates guinea-pig red cells (Baine, 1985), which are particularly rich in phosphatidylcholine (Turner et al., 1958). Accordingly, Dallas IE was chosen as the first strain of *L. pneumophila* from which to attempt isolation of phospholipase C.

**METHODS**

**Bacterial strain, media and growth conditions.** A subculture of the Dallas IE strain obtained from the Centers for Disease Control, Atlanta, Ga, USA, was maintained by serial passage on buffered charcoal/yeast extract agar (Feeley et al., 1979; Pascuelle et al., 1980). Purity of cultures was monitored by typical growth on buffered charcoal/yeast extract agar and failure to grow on buffered charcoal/yeast extract lacking a supplement of l-cysteine. HCl. Cultures in 25 ml portions of buffered yeast extract broth (Baine, 1985) were incubated at 21 °C in a rotary water bath at 180 r.p.m. A 10 ml volume of an 18 h broth culture was used to inoculate a Microferm Fermentor (New Brunswick) containing 8-0 l buffered yeast extract broth in which the yeast extract content was reduced to 2.5 g 1-1 to minimize foaming. The fermenter culture was incubated at 37 °C with agitation at 200 r.p.m. and aeration at 1-01 min-1. After 48 h the stationary-phase culture was brought to 3-0 mm with NaN3, and the cells were sedimented by centrifugation (4900 g, 30 min).

**Phospholipase C assay.** Phospholipase C activity was assayed by release of p-nitrophenol from p-nitrophosphorylcholine (NPPC) (Kurioka & Matsuda, 1976). Assay mixtures containing an appropriate volume of test sample, 5-0 mm-CaCl2, 5-0 mm-MnCl2, 3-0 mm-NaNO3, 2.5 mm-NPPC (Sigma), and 0.5-0% (v/v) Triton X-100 in 50 mm-HEPES (pH 7.5) at a final volume of 200 μl were placed in a water bath at 37 °C. The assay conditions were chosen on the basis of results of preliminary studies with crude preparations. Substrate control tubes substituting 50 mm-HEPES (pH 7.5) containing 3 mm-NaNO3, and a blank tube substituting buffer for the test sample, and sample control tubes substituting buffer for NPPC, and a blank tube substituting buffer for both the sample and NPPC were processed in parallel. After incubation, the mixtures were diluted with water or buffer and the A410 measured. Net A410 attributable to release of p-nitrophenol was determined from the reading in the diluted reaction mixture by subtracting the readings in the diluted sample and substrate control mixtures. The corresponding concentration of p-nitrophenol was calculated from a regression equation (HP-11 C, Hewlett-Packard) for a standard curve of A410 constructed using p-nitrophenol solutions of known molarity. This concentration was multiplied by the appropriate factor to calculate the molarity of p-nitrophenol present in the undiluted reaction mixture.

**Enzyme purification.** All but a 20 ml sample of 7-8 l of unfiltered culture supernate was applied onto a 5-0 cm-diameter column containing 120 ml DEAE-Sephadex (Pharmacia) previously equilibrated (Himmelhoch, 1971) at 4 °C with HEPES/NaNO3 buffer. Eluate was collected by gravity flow at this and subsequent stages of ion-exchange chromatography. The column bed was then washed with 100 ml HEPES/NaNO3 buffer and then with 50 ml 0.1 M-NaCl in the same buffer. Adsorbed material was then eluted with a linear gradient (BRL Gradient Former) from 0.1 to 0.3 M-NaCl in 180 ml HEPES/NaNO3 buffer followed by 130 ml 0.3 M-NaCl in the same buffer. The NaCl concentration in eluate fractions (5-0 ml) was monitored with a CDM 2e conductivity meter (Radiometer, Copenhagen).

Enzyme activity in each fraction was assayed as described above using 100 μl samples of eluate with incubation for 18 h. Sample control tubes were omitted since absorbance by the diluted eluate fractions was considered to be negligible. Fractions encompassing the peak of activity were pooled, and 91 of the resulting 101 ml were dialysed at 4 °C for 2 h against 10 vols 32 mm-MnCl2 in HEPES/NaNO3 buffer (Heppel, 1955) in tubing (Spectrum Medical Industries) with a cutoff for proteins of M, 12000–14000. Precipitated material was sedimented by centrifugation (3600 g, 30 min), and 82 of the resultant 92 ml of supernate were brought to 40% saturation with ammonium sulphate (Green & Hughes, 1955) in HEPES/NaNO3 buffer. After 5 min at 4 °C, the precipitate was sedimented by centrifugation as above, and 121 of the resultant 131 ml of supernate were dialysed at 4 °C against three changes (2 h, 2 h, 18 h) of 10 vols HEPES/NaNO3 buffer.

Phospholipase C in 150 μl volumes of samples from successive stages of enzyme purification was assayed by hydrolysis of NPPC as described above except that the manganese chloride supernate was assayed at 50.5 mm-MnCl2.
Protein concentrations were determined by the Bio-Rad Protein Assay microassay procedure, using bovine γ-globulin as the standard.

SDS-PAGE. Samples were concentrated by vacuum dialysis against 50 mM-Tris/HCl (pH 6.8) in a collodion bag apparatus (Schleicher & Schuell) and brought to 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.002% (w/v) bromophenol blue in 56 mM-Tris/HCl (pH 6.8). After heating at 100°C for 3 min and centrifugation (12000 g) to remove insoluble precipitates, the samples were subjected to SDS-PAGE (Hames, 1981) in 25 mM-Tris, 192 mM-glycine (pH 8.3) with a stacking gel of 3.8% (w/v) acrylamide in 125 mM-Tris/HCl (pH 6.8) and a linear-gradient resolving gel of 10–15% acrylamide in 375 mM-Tris/HCl (pH 8.8). Stacking was achieved at 120 V with resolution at 200 V. Haemophilus influenzae type b major outer-membrane protein (39000) (Gulig & Hansen, 1985) and proteins from M. kits (Bio-Rad), including myosin (200000), β-galactosidase (116250), phosphorylase b (92500), BSA (66200), ovalbumin (45000), carboxy anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (144000) were used as M, standards. Gels were stained with Coomassie blue R250 (Hames, 1981) or with a silver stain (Bio-Rad).

Confirmation of phospholipase C activity. Hydrolysis of authentic phosphatidylcholine, as distinct from NPPC, by the putative phospholipase C was demonstrated by release of 3H-label from lecithin-bearing tritiated choline, as previously described (Baine, 1985; Grossman et al., 1974). Dispersal of L-α-dipalmitoylphosphatidylcholine in HEPES/NaNO buffer, 74% mm–CaCl2, 0.32% (v/v) Triton X-100 was accomplished by vortexing for 2 min followed by sonication for 10 min using a Sonicator cell disrupter model W 185 F (Heat Systems-Ultrasonics) with the power setting at 9. L-α-Dipalmitoyl.[choline-methyl-3H]phosphatidylcholine [specific activity 27 Ci mmol–1 (1 TBq mmol–1), New England Nuclear] was then added to the unlabelled lecithin and dispersed by a second sequence of vortexing and sonication. A 25 ml sample of the purified enzyme preparation was concentrated 60-fold by vacuum dialysis against HEPES/NaNO buffer. The final reaction mixtures contained 75 μg protein, 1.0 mm–L-α-dipalmitoylphosphatidylcholine with 50 nCi L-α-dipalmitoyl-[choline-methyl-3H]phosphatidylcholine, 50 mm–HEPES, 3.5 mm–CaCl2, 3.0 mm–NaNO and 0.16% (v/v) Triton X-100 in a final volume of 200 μl. Substrate control tubes contained HEPES/NaNO buffer in place of enzyme. After 17 h incubation at 37°C, the reaction mixtures were acidified with 50 μl 6.84% (w/v) HClO4 and diluted with 250 μl distilled water. Unhydrolysed lecithin was removed from the mixture by serial extraction with 2 ml volumes of petroleum ether (boiling range 37–58°C), water-saturated diethyl ether and petroleum ether. Portions (200 μl) of the extracted aqueous phases were transferred to vials containing 10 ml Handifluor (Mallinkrodt) for liquid scintillation counting (Minaxi TRI-CARB 4000 series, Packard). Duplicate samples from substrate control tubes were also processed in parallel omitting the extractions with hydrophobic solvents and yielded a mean and range of 5769 ± 89 c.p.m. after correcting for background counts. Serial extraction with petroleum ether, diethyl ether and petroleum ether removed a mean and range of 56.1 ± 6.8% of the label from substrate control tubes, suggesting that the lipid solvents were only partially successful in extracting phosphatidylcholine from the aqueous suspension or that some of the label was present on polar decomposition products of the tritiated lecithin. The proportion of tritiated phosphorylcholine released into the acidified aqueous phase was calculated as follows after correcting for background counts: (c.p.m. in extracted aqueous phase of test – c.p.m. in extracted aqueous phase of substrate control)/c.p.m. in unextracted substrate control.

Effect of substrate concentration. Enzyme activity in the purified preparation was assayed by hydrolysis of NPPC as described above, except that the substrate concentration varied from 5 to 100 mm–NPPC. The values for −Km and Vmax were taken as the slope and ordinate intercept, respectively, of the linear regression of the data (HP-11C, Hewlett-Packard) on a Woelfl–Augustinsson–Hofstee plot, which is not strongly affected by small errors in the measurement of V at low substrate concentration (Segel, 1976). Reaction mixtures contained 1–1 μg protein and were incubated for 30, 61 and 170 min in three separate experiments.

pH optimum. Enzyme activity in the purified preparation as a function of pH was determined by hydrolysis of NPPC in the standard assay, except that 3.0 mm–NPPC was used as substrate and the reaction was done in 0.3 mm–HEPES over a range from pH 6.51 to pH 8.46 with corresponding sample and substrate control tubes. The reaction mixtures contained 1.6 μg protein and were incubated for 60 min. Measurements of p-nitrophenol absorbance were made at each pH to give appropriate standard curves.

Divalent cation requirements. Dialysed ammonium sulphate supernate was brought to 1.0 mm–EDTA and then dialysed at 4°C over 6 h against three changes of 10 vols HEPES/NaNO buffer. Phospholipase C activity of the chelated preparation was determined by hydrolysis of NPPC as described for the standard assay, except that 0.0 mm–NPPC was used as substrate, and the usual combination of CaCl2 and MnCl2 was replaced by incremental concentrations of the chloride salts of eight different divalent cations. The reaction mixtures contained 1.9 μg protein and were incubated for 65 min.

Effects of detergents. The effects of various detergents on enzyme activity in the purified preparation were determined by hydrolysis of NPPC in 50 mm–HEPES (pH 7.5) as described for the standard assay, except that 10 mm–NPPC was used as substrate and 0.50% (v/v) Triton X-100 was omitted or replaced by one of six different detergents. These were present at only 0.25% (w/v) to minimize the risk that differences in the effects of the
detergents might be obscured if they were present in excess. The reaction mixtures contained 1·7 μg protein and were incubated for 80 min.

Effects of sugar alcohols. The effects of 40% (w/w) glycerol and of 33% (w/w) sorbitol on enzyme activity in the purified preparation were determined by hydrolysis of NPCC as described for the standard assay after addition of the appropriate sugar alcohol. The reaction mixtures contained 1·1 μg protein and were incubated for 170 min.

Test for haemolytic activity. Petri dishes containing 20 ml dog-blood agar were prepared with 5% (v/v) sterile defibrinated dog blood and 3·9% (w/v) tryptose agar (Difco) (Baine et al., 1979). Haemolytic activity in samples from successive stages of enzyme purification was sought by inoculating 150 μl volumes of each preparation into wells 9 mm in diameter that had been bored into the agar. The plate was incubated for 18 h at 37 °C and then for 4 h at 4 °C with periodic inspection for zones of haemolysis around the wells.

HEPES/NaN3 buffer was brought to 86 mM-NaCl, and a sample of the purified enzyme preparation was brought to 53 mM-NaCl to render the solutions isotonic (267 mOsm kg−1 and 262 mOsm kg−1, respectively, by freezing-point depression). Serial twofold dilutions of the purified enzyme preparation were made in the isotonic buffer in a microtitre plate. Duplicate rows of wells were supplemented with Tween 80, with or without CaCl2, and with or without MnCl2. After addition of defibrinated dog blood, each well contained the purified preparation at dilutions of 1 in 2 through 1 in 2048 and 2·5% (v/v) dog red cells and, as appropriate, 0·025 mM-CaCl2, and 0·025 mM-MnCl2 in isotonic HEPES/NaN3 buffer in a final volume of 100 μl. Control wells contained isotonic buffer in place of the purified enzyme. The plate was incubated for 60 min at 37 °C and then for 18 h at 4 °C and periodically inspected for haemolysis.

RESULTS

Enzyme purification. Maximal NPPC-hydrolysing enzyme activity eluted from the DEAE-Sephadex column in the range of 6·9–11·1 mS (110–207 mM-NaCl) (Fig. 1). Assessment of protein concentration in the fractions by measurements of A280 was confounded by absorbance by a brown pigment (Baine & Rasheed, 1979; Baine et al., 1978) in the supernate.

The precision of the NPPC assay of phospholipase C activity in unconcentrated culture
supernate was limited by the prolonged period required to demonstrate significant hydrolysis of this substrate by such a dilute solution of enzyme. However, the specific activity was increased approximately 50-fold by ion-exchange chromatography and manganous chloride fractionation of the supernate (Table 1). Ammonium sulphate fractionation increased the purity of the preparation, as determined by SDS-PAGE (Fig. 2), but actually resulted in some loss of specific activity. Assay of the supernate obtained after equilibration with 52 mM-MnCl₂ was done at a relatively high concentration of Mn²⁺, so the paradoxical decline in specific activity after ammonium sulphate fractionation could have been an artifact of differing concentrations of Mn²⁺ in the assay mixtures. However, preliminary experiments (data not shown) indicated that whereas enzyme activity could be removed from manganous-chloride-treated DEAE-Sephadex eluates by ammonium sulphate at 60% saturation, recovery of enzyme activity from the resulting precipitate was poor.

Significant enzyme activity was present in DEAE-Sephadex eluate fractions that were not pooled for further processing (Fig. 1). Artifacts possibly contributing to the high apparent recovery of enzyme during purification could include imprecision in the assay of weak phospholipase C activity in unconcentrated supernate as well as removal of enzyme inhibitors and alteration in relevant ion concentrations during purification.

After SDS-PAGE crude culture supernate stained with Coomassie blue revealed a faint band with an M₉ of 50000 and a major band with an M₉ of 38000 (Fig. 2). After DEAE-Sephadex ion-exchange chromatography, the 50 kDa band was the dominant protein by Coomassie-blue staining. Further purification by treatment with manganous chloride and ammonium sulphate yielded one visible band only by Coomassie-blue staining. In two SDS-PAGE gels this protein migrated with an apparent M₉ of 50000 and 54000.

**Hydrolysis of authentic phosphatidylcholine.** Purified enzyme released a net of 33-7% [(4475 c.p.m. – 2533 c.p.m.)/(5769 c.p.m.)] ± 1-0% (mean ± range) of the total amount of label present in tritiated lecithin into the aqueous phase as presumed phosphorylcholine.

**Phospholipase C kinetics.** Phospholipase C activity increased with increasing substrate concentration over the range from 5 to 100 mM-NPPC. The enzyme showed a low affinity for NPPC, with an apparent Kₐ of 35, 51 and 61 mM for this artificial substrate in three separate experiments. The corresponding calculated values of V₉₉₉ for hydrolysis of NPPC were 4-3, 5-0 and 4-6 μmol l⁻¹ min⁻¹ at 37 °C.

**pH optimum for enzyme activity.** Phospholipase C activity increased with increasing alkalinity from pH 6-51 through pH 8-46 (Table 2). Accurate measurement of enzyme activity at lower pH was impeded by inaccuracy in measuring very low concentrations of p-nitrophenol at acid pH, at which the molar extinction coefficient of this compound is low.
Fig. 2. SDS-PAGE of phospholipase C preparations from *L. pneumophila* (Dallas 1E) at different stages of purification on 10–15% linear polyacrylamide gradient gel stained with Coomassie blue. Lane A, crude culture supernate (31 μg protein); lanes B (34 μg) and E (68 μg), pooled DEAE-Sephadex eluate fractions 25–45; lanes C (16 μg) and G (31 μg), 52 mM-MnCl₂ precipitation supernate; lanes D (19 μg) and I (38 μg), supernate of 40% saturation ammonium sulphate precipitation; lanes F and H, blanks containing 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.002% (w/v) bromophenol blue in 62.5 mM-Tris/HCl (pH 6.8). The positions of *M* standards are noted.

**Divalent cation requirements for enzyme activity.** Addition of 1–10 mM-Ba²⁺, Ca²⁺, Mn²⁺ or Mg²⁺ to EDTA-treated purified enzyme produced a dose-dependent enhancement of phospholipase C activity (Fig. 3). The more strongly oxidizing Zn²⁺, Cu²⁺ and Fe²⁺ (De Vries, 1961) at concentrations as low as 0.01–1 mM inhibited the enzyme. Although the electrochemical potential of Co²⁺ makes this ion a stronger oxidizer than Zn²⁺ or Fe²⁺, Co²⁺ was comparable to Mg²⁺ in enhancing the activity of the chelated enzyme preparation.

**Effects of detergents upon enzyme activity.** Little effect on enzyme activity was observed with 0.25% (w/v) Nonidet P-40, but the other nonionic detergents that were tested significantly enhanced hydrolysis of NPPC (Table 2). The polysorbate detergents Tween 20 and Tween 80 were most effective, but Triton X-100, a polyethylene glycol p-isooctylphenyl ether, also significantly accelerated hydrolysis of the substrate in comparison with detergent-free control mixtures. Sodium deoxycholate, a bile salt, had little effect on enzyme activity, whereas the ionic detergent SDS was strongly inhibitory.

**Effects of sugar alcohols upon enzyme activity.** Sorbitol at 33% (w/w) enhanced enzyme activity over sugar-alcohol-free mixtures, but no effect was observed with 40% (w/w) glycerol (Table 2).

**Screen for haemolytic activity.** No lysis of red cells was apparent in the radial haemolysis assay in dog-blood agar. No difference between control wells and those containing the purified enzyme was apparent in the microtitre haemolysis assay.
Fig. 3. Effects of divalent cations on activity of EDTA-treated purified phospholipase C from *L. pneumophila* (Dallas 1E). One unit (U) is defined as 1 nmol p-nitrophenol released by hydrolysis of NPPC min⁻¹ at 37 °C. Values shown are the means and ranges of duplicate assays. Ions are grouped as Ba²⁺ (△), Ca²⁺ (●) and Zn²⁺ (○); Co²⁺ (△), Fe²⁺ (●) and Cu²⁺ (●); and Mn²⁺ (○) and Mg²⁺ (△).

Table 2. Effect of pH, detergents and sugar alcohols upon activity of phospholipase C from the Dallas 1E strain of *L. pneumophila* serogroup 5

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<th>pH</th>
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* One unit is defined as 1 nmol p-nitrophenol released by hydrolysis of NPPC min⁻¹ at 37 °C. Values are means and ranges of duplicate assays.

**DISCUSSION**

SDS-PAGE indicated that an increase in purity of the phospholipase C from the Dallas 1E strain of *L. pneumophila* was achieved at each step of the sequence of ion-exchange chromatography, manganous chloride fractionation and ammonium sulphate fractionation.
Nevertheless, despite an improvement in purity, the specific activity of the preparation apparently declined after exposure to ammonium sulphate at 40% saturation. Plausible explanations for this paradox include the effect of a change in Mn$^{2+}$ concentration on enzyme activity as well as the possibility that the enzyme was partially inactivated by transient exposure to ammonium sulphate (Green & Hughes, 1955).

Recovery of the enzyme from culture supernates does not exclude the possibility that there are cell-associated stores of this protein. However outer-membrane protein profiles of the Dallas 1E strain published by Hindahl & Iglewski (1986) did not show a prominent band at 50–54 kDa. No analogous information is available on proteins in the periplasmic space and cytosol.

Hydrolysis of NPPC is in common use as an assay for phospholipase C, but a phosphodiesterase might also be able to release $p$-nitrophenol from the same artificial substrate (Krug & Kent, 1981). Phospholipase C activity in the purified preparation from the Dallas 1E strain was confirmed by release of label from authentic lecithin bearing tritiated choline. This label is presumed to have been in the form of tritiated phosphorylcholine. However, if choline were not extracted from the acidified mixture by petroleum ether and diethyl ether, hydrolysis of the tritiated lecithin by a phospholipase D might also have been detected by this assay.

The observed specificity and activity of a given phospholipase are conditioned by the identities of the fatty acid moieties and of the polar group present on the substrate (Dennis, 1983; El-Sayed et al., 1985). Furthermore, in contrast to simpler systems involving enzymes acting upon water-soluble substrates, the target of a phospholipase may be present as monomer or aggregated in micelles, reverse micelles, mixed micelles, or unilamellar or multilamellar vesicles. This variability in the physical state of a phospholipid substrate can also affect the apparent specificity and activity of a given enzyme (Mollby, 1978; Dennis, 1983).

Substituting NPPC for native phospholipid simplifies the system by eliminating the structure and physical state of the lecithin target as variables. The soluble substrate also permits analysis using classical Michaelis-Menten kinetics, which are inappropriate for native phospholipids employed at levels above their critical micellar concentrations (Waite, 1985). However, data on the activity of an enzyme upon this artificial substrate should not be extrapolated uncritically to enzyme–substrate interactions with native phospholipids in vivo.

The high apparent $K_m$ for NPPC of the purified phospholipase C from the Dallas 1E strain was comparable to that (0.2 M) reported for phospholipase C from Clostridium perfringens (Kurioka & Matsuda, 1976). El-Sayed et al. (1985) reported that the affinity of phospholipase C from Bacillus cereus decreased when the fatty acid chain length in the substrate was less than six carbons. The absence of fatty acids in NPPC may have contributed to the high $K_m$ observed with this analogue of phosphatidylcholine.

The specific activity of purified phospholipase C from the Dallas 1E strain was maximal at alkaline pH. L. pneumophila is a nonfermentative organism (Hoffman, 1984), and its growth in broth cultures (Pine et al., 1979) or within the phagosomes of human monocytes (Horwitz & Maxfield, 1984) is accompanied by an increase in the pH of its environment. Such alkalinization should favour activity of the phospholipase C.

Various bacterial phospholipases C are metalloenzymes susceptible to inactivation by EDTA and to enhancement or inhibition by supplementation with various divalent cations (Kurioka & Matsuda, 1976; Möllby, 1978; Dennis, 1983). Activity of the purified enzyme from the Dallas 1E strain was also potentiated by several divalent cations and inhibited by others. Although Zn$^{2+}$ enhances the activity of phospholipase C from C. perfringens at concentrations up to $10^{-4}$ M, the Dallas 1E enzyme was strongly inhibited by this cation.

Hydrolysis of native lecithin by phospholipase C may be markedly affected by the presence of certain detergents which affect the physical state of the substrate (Dennis, 1983; El-Sayed & Roberts, 1985). Although NPPC is freely soluble in water, the activity of phospholipase C from C. perfringens upon this lecithin analogue is enhanced by strong ionic detergents, presumably through interactions with the enzyme instead of the substrate (Kurioka & Matsuda, 1976). In contrast, nonionic detergents enhanced the activity of the phospholipase C of L. pneumophila, whereas a strong ionic detergent was inhibitory.

Kurioka & Matsuda (1976) reported enhancement of hydrolysis of NPPC by phospholipase C
from *C. perfringens* in the presence of high concentrations of sorbitol or glycerol. Sorbitol also enhanced the activity of the purified enzyme from the Dallas 1E organism, but no effect of glycerol on the reaction was documented.

Although it is possible that conditions exist under which this phospholipase C might damage erythrocyte membranes (Möllby, 1978), no evidence of ability to lyse red cells was noted.

The substrate range and antigenicity of this phospholipase C remain to be determined, as does the degree of variability of the enzyme among strains and species of *Legionella*. Finally, the extent to which phospholipase C plays a role in the pathogenesis of legionellosis remains to be elucidated.

This work was presented in part at the Twenty-sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, held in New Orleans, Louisiana, on September 28–October 1, 1986.

I thank Laurinda Ng, Prakuzy Sebastian, Colleen Kennedy and Edward Davis for very capable technical assistance. I am grateful to Robert Munford, Leon Eidsel and Eric Hansen for good advice and to Dr Hansen for a generous gift of *H. influenzae* type b major outer-membrane protein.

REFERENCES


