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A New Kinetic Approach for Studying Phospholipase C (*Clostridium perfringens* α Toxin) Activity on Phospholipid Monolayers†

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ABSTRACT: The enzymatic activity of purified phospholipase C (α toxin) from *Clostridium perfringens* was investigated with various phospholipid monolayers. A two-step reaction was used. Enzymatic hydrolysis of insoluble lecithin films by phospholipase C, generating 1,2-diacylglycerol and water-soluble phosphocholine, was coupled with the action of pancreatic lipase in order to give rise to fatty acid and 2-monoacylglycerol, which are rapidly desorbed from the interface. With this new procedure, it is possible to obtain continuous and accurate kinetic measurements of the phospholipase C catalyzed reaction with phospholipid monolayers as the substrate. It is thus possible to avoid the use of radiolabeled substrates as necessary in previous studies, and the difficulties caused by diacylglycerol accumulation in the lipid film are minimized. No hydrolysis was detected when either phosphatidylethanolamine, phosphatidylserine, or phosphatidylglycerol films were used as substrates. By means of a film transfer technique, Ca^{2+} and Zn^{2+} ions were found to play a specific and critical role. The present study demonstrates clearly for the first time that Ca^{2+} is essential for enzyme binding to lipid films, whereas Zn^{2+} is specifically involved in the catalytic hydrolysis of the substrate.

Clostridium perfringens α toxin is generally described as being lethal, dermonecrotic, and hemolytic (McDonel, 1980). It is known to be the main toxin of *Clostridium perfringens* and to constitute a major pathogenic factor in the development of gas gangrene and septicemia (McDonel, 1980).

Since the enzymatic nature of α toxin (phospholipase C) was discovered by McFarlane and Knight (1941), most studies dealing with its mode of action have used assays involving either detergent-solubilized phospholipid substrates (Yamakawa & Ohsaka, 1977) or erythrocyte lysis (Taguchi & Ikezawa, 1976).

Phospholipid hydrolysis by phospholipase C leads to the production of water-insoluble diacylglycerols and water-soluble phosphocholine. In previous investigations, kinetic studies were performed with phospholipase C on monomolecular film with ^{32}P-labeled phospholipids (Bangham & Dawson, 1962; Hirasawa et al., 1981) or [^{3}H]choline- or [^{14}C]choline-labeled phospholipids (Miller & Ruysschaert, 1971; Demel et al., 1975). During hydrolysis by phospholipase C, the solubilization of radiolabeled phosphocholine in the water subphase leads to a decrease in the surface film radioactivity. However, diacylglycerol accumulation with time results in a decrease in the phospholipid surface density. Furthermore, the lipid composition of the monolayer varies during the experiment, making it difficult to interpret the kinetics. In order to minimize the problems associated with product accumulation, we have developed a new procedure where the diacylglycerols formed during phospholipase C action are hydrolyzed by the action of a pancreatic lipase into fatty acid and 2-monoacylglycerol, which are then desorbed from the interface. It has been shown previously that pancreatic lipase does not

† This is paper 10 in a series on enzyme reactions in a membrane model. For paper 9, see Gargouri et al. (1986). This study is part of the doctoral thesis obtained by H.M. at Paris VII University, June 25, 1985. The key concepts were presented at the second European Workshop on Bacterial Protein Toxins in Wepion, Belgium, June 30, 1985, to July 4, 1985.

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significantly hydrolyze pure PC monolayers (Verger et al., 1976).

Materials and Methods

Enzymes. Phospholipase C from the culture supernatant of *Clostridium perfringens* type A, strain N5, was purified to apparent electrophoretic homogeneity (SDS–PAGE)[1] (Moreau et al., unpublished experiments). Briefly, a 4-h culture in a fermentor maintained at pH 7.6 was supplemented with cholesterol (20 mg/mL) and centrifuged, and the supernatant was precipitated with $(NH_4)_2SO_4$ to 60% saturation. The precipitate dissolved in pH 7.2 buffer was gel filtered on Bio-Gel P-100 (Bio-Rad Laboratories) and the active pool subjected to preparatory isoelectric focusing in a pH 3.5–11 gradient in a 110-mL column (LKB). The fraction focusing into a sharp peak with p*I* of 5.5 constituted the purified phospholipase C (α toxin). The molecular mass was 43 kDa, and the specific activity was 250 units/mg. One unit was the amount of enzyme which liberates 1 μmol of H^+ per minute with egg PC as substrate in a titrimetric procedure.

Pig pancreatic lipase was purified at the laboratory as described by Verger et al. (1969) and was saturated by colipase addition.

Lipids. Synthetic 1,2-didodecanoyl-*sn*-glycero-3-phospho-X (X = choline, ethanolamine, serine, and glycerol) were gifts from Dr. Slotboom (Utrecht, The Netherlands). Egg PC was purchased from Sigma, cholesterol was from Fluka, and EDTA and phosphatidic acid were from Merck. The lipids were homogeneous as checked by thin-layer chromatography.

Solutions of pure lipids were prepared by weighing a dry sample to which was added a known weight of chloroform.

Monomolecular films were obtained by spreading a lipid solution onto a clean air/water interface with a micrometer-controlled Agla syringe (Burroughs-Wellcome Laboratories, U.K.).

Monitoring Reaction Rates. The surface barostat method has been described elsewhere (Verger & De Haas, 1973). We used a zero-order trough composed of a reservoir 17.6 × 28.4 cm in size with a reaction compartment containing the enzymes (total volume 210 mL; total surface area 123 cm^2). All experiments were performed at 25 °C. The subphase of the reaction compartment was stirred with two magnetic stirrers (250 rpm). The following buffer was used throughout the experiments: 0.15 M NaCl; 5 mM $CaCl_2$; 0.1 mM $ZnSO_4$; 20 mM Tris-HCl; pH 7.2. Before each experiment, the trough was cleaned with ethanol and rinsed several times with tap water until the Teflon surface no longer retained any water drops. It was finally rinsed twice with distilled water.

Hydrolysis of Mixed PC/Cholesterol Monolayers. Mixed films of PC/cholesterol films were obtained as described previously (Pieroni & Verger, 1979). A Teflon barrier was placed across the small channel of the zero-order trough between the reservoir and the reaction compartment in order to block the surface communication. The surface pressure was initially determined by placing the platinum plate in the reaction compartment where the mixed film was spread at the desired pressure. The surface pressure was then measured after the platinum plate was transferred to the reservoir compartment where the pure PC substrate film was subsequently spread. Surface pressure was adjusted to the value in the reaction compartment by displacing the mobile barrier, and the barrier between the two compartments was then removed to allow communication between the two monomolecular films. The pressure change during these operations did not exceed 0.25 mN/m. The enzyme was then injected into the reaction compartment, and the kinetics were recorded as described by Verger and De Haas (1973).

Film Transfer. In some experiments the monomolecular film was transferred from one subphase to another as previously described (Rietsch et al., 1977). In order to perform these experiments, a special trough having three identical reaction compartments connected by surface channels was used (Rietsch et al., 1977).

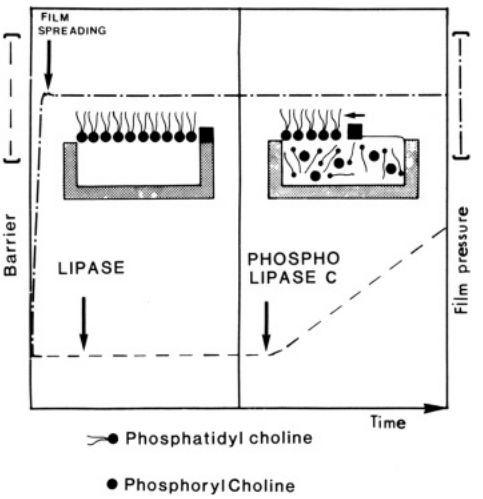

FIGURE 1: Principle of the phospholipase C activity determination method using phospholipid monolayers as substrates.

Results

Two Enzymatic Step Approach to Phospholipase C Activity Measurement. A pure synthetic phospholipid monolayer of PCdiC12 was spread at the air/water interface and maintained at a constant surface pressure of 14 mN/m. Pancreatic lipase saturated with colipase was added to the aqueous subphase, and no change in surface pressure was detected during 10 min (Figure 1). Phospholipase C was then added, and the reaction process, as reflected by the barrier movement of the barostat, was recorded as shown schematically in Figure 1. With the same phospholipase C concentration (40 pM final concentration), we obtained identical results using different lipase amounts ranging from 0.15 to 10 nM final concentrations (data not shown). The rate of PCDiC10 or PCDiC12 monolayer hydrolysis at a constant surface pressure of 14 mN/m was measured at various concentrations of phospholipase C in the presence of a fixed concentration (1 nM) of lipase saturated with colipase. With both substrates, the phospholipid hydrolysis rates were found to be linearly dependent upon a phospholipase C concentration ranging from 0 to 70 pM (data not shown). When PCdiC10 was used as substrate instead of PCDiC12, the rate of hydrolysis was found to be 2.8 times higher (data not shown).

Influence of Film Pressure. The phospholipase C reaction rate as a function of surface pressure gave a bell-shaped curve with PCdiC10 and PCdiC12 films as substrates (Figure 2). With both substrates, the optimal surface pressure was around 15 mN/m, but at all surface pressures tested, PCdiC10 was hydrolyzed at about twice the rate of PCdiC12. However with both substrates, the enzyme activity decreased sharply near

[1] Abbreviations: BSA, bovine serum albumin; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PCdiC10, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; PCdiC12, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine; PEdiC12, 1,2-didodecanoyl-*sn*-glycero-3-phosphoethanolamine; PSdiC12, 1,2-didodecanoyl-*sn*-glycero-3-phosphoserine; PGdiC12, 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

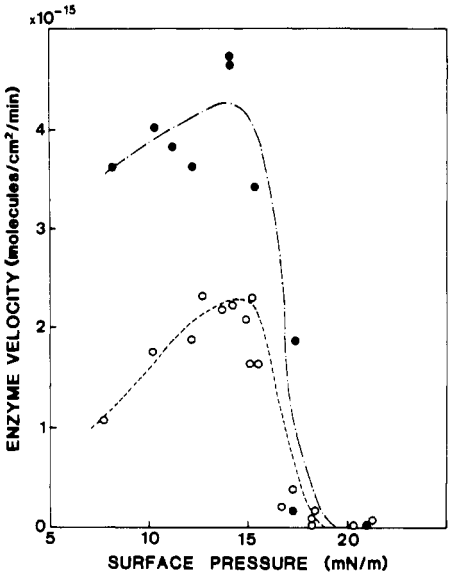

FIGURE 2: Phospholipase C activity as a function of surface pressure, using PCdiC10 (●) and PCdiC12 (○) as substrates. Pancreatic lipase was added to the subphase at a final concentration of 1 nM and phospholipase C at 40 pM final concentration. Buffer was 20 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.1 mM $ZnSO_4$, pH 7.2.

a surface pressure threshold of around 15–17 mN/m (Figure 2). Phospholipase C (40 pM) was injected 10 min after pancreatic lipase (1 nM) saturated with colipase under a PCdiC12 monolayer at 18 mN/m. No hydrolytic activity was observed. Then the film was transferred at constant surface pressure over the surface of another compartment containing pancreatic lipase saturated with colipase. The PCdiC12 film was then decompressed from 18 to 11 mN/m, and no enzymatic activity was detected. As a control experiment we performed a film transfer at a constant surface pressure of 11 mN/m under the same conditions. In the latter case, full enzymatic activity was recorded after the film transfer (data not shown). These two experiments clearly indicate that phospholipase C did not associate with the lipid film at a surface pressure above the threshold of 17 mN/m. Since it was previously observed that porcine pancreatic lipase binds to mixed glyceride/PCdiC12 films up to 22 mN/m (Pieroni & Verger, 1983), the lack of phospholipase C activity at surface pressures higher than 17 mN/m can be attributed to the fact that the phospholipase C exhibits no interfacial binding.

Action of Phospholipase C on PC/Cholesterol Mixed Films. In order to mimic the lipid composition of an erythrocyte membrane, we studied the influence of cholesterol on phospholipase C action. Mixed films composed of PCdiC12 and cholesterol with molar ratios of 1 and 1/2 were used. Activity versus surface pressure profiles showed a significant increase in the pressure threshold due to the presence of cholesterol as compared to pure PCdiC12 film (Figure 3).

To investigate the effect of cholesterol on long-chain PC film hydrolysis, we used mixed egg PC/cholesterol films (molar ratio 1/2), with BSA (4 μM final concentration) in the subphase so that long-chain reaction products would be solubilized. When pure egg PC monolayers were used (Figure 4), enzymatic activity was detected up to a surface pressure of 30 mN/m. The presence of cholesterol in the PC film increased the threshold pressure from 30 to 35 mN/m. This was the highest pressure at which any enzymatic activity was still detectable. Below 22 mN/m, it was impossible to detect any enzymatic activity, due to the high tensioactivity of BSA (Verger & Pieroni, 1986).

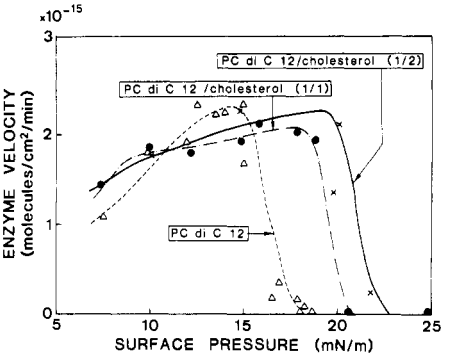

FIGURE 3: Phospholipase C activity as a function of surface pressure using pure PCdiC12 (△) films and mixed PCdiC12/cholesterol films at molar ratios of 1/1 (●) and 1/2 (×). Pancreatic lipase was added to the subphase at a final concentration of 1 nM and phospholipase C at 40 pM final concentration. Buffer was 20 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.1 mM $ZnSO_4$, pH 7.2.

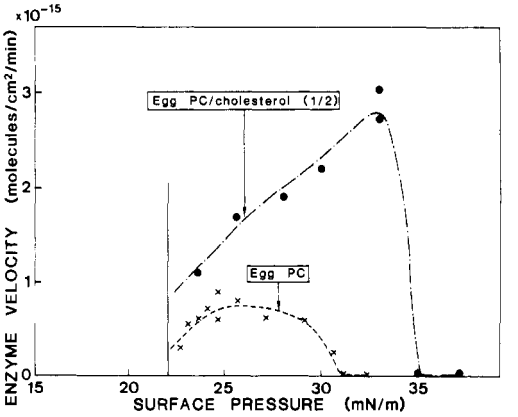

FIGURE 4: Phospholipase C activity as a function of surface pressure, using egg PC films (×) and mixed egg PC/cholesterol films at a molar ratio of 1/2 (●). BSA was added to the subphase at 4 μM final concentration and pancreatic lipase at 1 nM and phospholipase C at 40 pM final concentrations. Buffer was 20 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.1 mM $ZnSO_4$, pH 7.2.

Substrate Specificity. Phospholipase C (40 pM) activity was tested with as substrate synthetic PCdiC12, PSdiC12, PEdiC12, or PGdiC12 maintained at a constant surface pressure of 10 mN/m. Enzymatic activity could be detected only when PCdiC12 films were used. No significant activity was observed with the other phospholipids tested, even after the addition of phosphatidic acid to the monolayers (data not shown). Thus, under our experimental conditions, negatively charged phospholipids are not substrate for phospholipase C of *C. perfringens*.

Effect of Calcium and Zinc Ions. When EDTA (0.1 mM) was introduced into the aqueous subphase of the reaction compartment, phospholipase C activity, measured under optimal conditions, was immediately and completely abolished although under these conditions pancreatic lipase is known to be fully active (Rietsch et al., 1977). Hydrolysis was not restored after addition of either Zn^{2+} (0.5 mM final concentration) or Ca^{2+} (5 mM final concentration) alone. The simultaneous presence of the two cations in the aqueous subphase was necessary for the activity of the phospholipase C to be restored (data not shown).

To determine the respective roles of Ca^{2+} and Zn^{2+} ions on phospholipase C activity, transfer experiments were performed as described in Figure 5. Lipase (1 nM final concentration) saturated with colipase was added to all three compartments. The portion of the film located over compartment 1, where phospholipase C was first injected, was isolated by barrier B and transferred to the surface of compartment 3 with a film

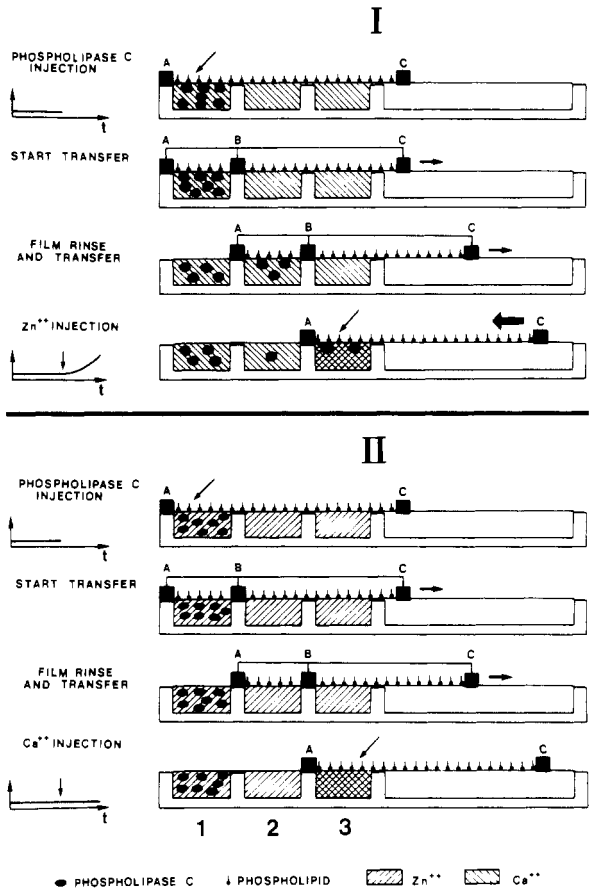

FIGURE 5: Transfer experiments showing the role of Ca^{2+} and Zn^{2+} ions in phospholipase C activity. A, B, and C represent the three barriers; barrier C was mobile during the reaction recording. The graphs on the left side of the figure are the kinetic curves recorded before (upper graph in I and II) and after (lower graph in I and II) film transfer. Lipase saturated with colipase was present in all the reaction compartments. See Pieroni and Verger (1979) and Results for experimental details.

rinse over compartment 2. In experiment II, Zn^{2+} ions (0.5 mM final concentration) alone were first present in the three compartments, and after film transfer Ca^{2+} (5 mM final concentration) was injected into the aqueous subphase of compartment 3; no enzymatic activity was observed. As a control, a further injection of phospholipase C into the aqueous subphase of compartment 3 was found to initiate film hydrolysis at a comparable rate to that observed previously. In experiment I, Ca^{2+} ions (5 mM final concentration) were initially present in all three compartments. After film transfer, injection of Zn^{2+} (0.5 mM final concentration) into the aqueous subphase of compartment 3 initiated an immediate phospholipase C activity, amounting to 60% of the optimal value previously determined.

Discussion

Two Enzymatic Step Approach to Phospholipase C Activity Measurement. In the present study we developed a new approach with which continuous kinetic measurements of phospholipase C activity can be performed on phospholipid monolayers under controlled surface pressure. The basic principle involves coupling the phospholipase C reaction with the subsequent hydrolysis by means of a lipase of the diglycerides formed. We first checked that phospholipase C activity was independent of lipase and colipase concentration in the range of 0.15–10 nM. Furthermore, the fact that the rate of hydrolysis depends linearly on phospholipase C concentration in the presence of a constant lipase and colipase concentration (1 nM) supports our conclusion that lipase hydrolysis is not the rate-limiting step in the range of phospholipase C concentrations tested.

On the other hand, the addition of lipase and colipase underneath the PC monolayer was not followed by any detectable change in surface pressure. This indicates that no significant penetration of lipase and no hydrolysis of the phospholipid film by this enzyme occurred. From the results obtained at our laboratory by Gargouri et al. (1986), one can calculate that under our experimental conditions less than 2 ng/cm^2 of lipase are adsorbed to the PC monolayer. This amount represents a similar catalytic surface excess to that observed in previous studies using other lipid films (Momsen & Brockman, 1981; Pieroni & Verger, 1979).

The difference observed between the rates of hydrolysis of PCDiC10 and PCDiC12 (2.8-fold) cannot be explained by the difference between the molecular areas of these two compounds, which differ by less than 4% (Zografi et al., 1971).

Importance of Film Pressure. The same surface pressure threshold values (17 mN/m) were observed when phospholipase C action took place on PCdiC10 and PCdiC12 films (see Figure 3). PCDiC12 film transfer experiments below and above the pressure threshold demonstrate that the lack of enzyme activity is due to a lack of phospholipase C binding to the lipid monolayer. This threshold value corresponds approximately to a phospholipid molecular area of 65 Å^2/molecule for both molecules. When natural long-chain phospholipids (egg lecithin) were used as substrate, the penetration threshold was around 31 mN/m, corresponding to a phospholipid molecular area of 67 Å^2/molecule. A comparable surface pressure threshold value (29 mN/m) has been found by Demel et al. (1975) using radiolabeled palmitoyloleoylphosphatidylcholine. The existence of a critical molecular area has been observed previously with pancreatic phospholipase A_2 acting on various PC monolayers and mixed PC/triacylglycerols films (Pieroni & Verger, 1983). Mixed cholesterol/PC films were used in order to mimic the lipid composition of the erythrocyte membrane. The presence of cholesterol shifted the phospholipase C activity threshold to higher surface pressure values when either synthetic PCdiC12 or egg PC was used (see Figures 4 and 5). Similarly, Demel et al. (1975) have reported that mixed films of PC/cholesterol/sphingomyelin show higher threshold values than pure PC films.

It is noteworthy that mixed egg PC/cholesterol films (0.5 molar ratio) can be hydrolyzed by phospholipase C up to a surface pressure of nearly 35 mN/m. This value is comparable to those (31–35 mN/m) attributed by Demel et al. (1975) to the native erythrocyte membrane. It is thus understandable, in view of its high penetration power into mixed PC/cholesterol films, that pure phospholipase C from *Clostridium perfringens* should be a hemolytic toxin (Taguchi & Ikezawa, 1976; McDonel, 1980; Takahashi et al., 1981; Ikezawa et al., 1983).

Role of Calcium and Zinc Ions. It is well-known that phospholipase C activity cannot be detected in the absence of Ca^{2+} or Zn^{2+} ions (Klein, 1975; Takahashi et al., 1981), but the exact role of these two ions has not been clearly established. When only Zn^{2+} ions were present in the aqueous subphase, phospholipase C was not associated with the substrate film and consequently was not active. In the presence of Ca^{2+} ions only, phospholipase C was associated with the film, but its activity was dependent upon the presence of Zn^{2+} ions. Full enzymatic activity was expressed only when Zn^{2+} and Ca^{2+} ions were simultaneously present (see Figure 6). Ca^{2+} ions

appear to be involved in the binding of the enzyme to the lipid interface, as previously hypothesized (Takahashi et al., 1981), unlike Zn^{2+} ions, which are required for the expression of catalytic activity. These findings are compatible with data reported by Krug et al. (1984), working with atomic absorption spectrophotometery, who found two zinc atoms per phospholipase C molecule.

In the case of phospholipase A_2 from pancreas or snake venoms, the absolute and specific Ca^{2+} ion requirements seem to be due to their structural rather than their catalytic function (Verheij et al., 1981). It has also been suggested that Ca^{2+} ions play an important part in the lipid binding step of these enzymes (Verger et al., 1973; Pattus et al., 1979). From the present study, it seems clear that the binding of phospholipase C to lipid interfaces requires Ca^{2+} ions and that expression of its catalytic properties requires Zn^{2+} ions.

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Registry No. PCdiC10, 3436-44-0; PCdiC12, 18194-25-7; PEdiC12, 59752-57-7; PSdiC12, 76260-76-9; PGdiC12, 63644-55-3; Ca, 7440-70-2; Zn, 7440-66-6; phospholipase C, 9001-86-9; cholesterol, 57-88-5.

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