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Handbook of Lipid Research

The Phospholipases

Moseley Waite

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This book is dedicated to my two mentors

Salih J. Wakil

and

Laurens L. M. van Deenen

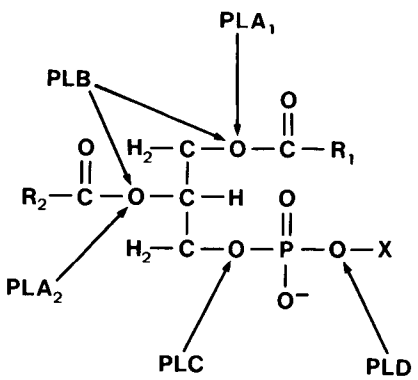
I owe much for the efforts they gave

And to my family

May they always find the white water to paddle

Preface

Phospholipases are a class of ubiquitous enzymes that have in common their substrate and the fact that they are all esterases. Beyond that, they are a diverse group of enzymes that fall into two broad categories, the acyl hydrolases and the phosphodiesterases. The former group is made up of the phospholipases A_1 and A_2, phospholipase B, and the lysophospholipases. On the other hand, the phosphodiesterases are the phospholipases C and D. The scheme indicates the site of attack of each type of phospholipase.



The lysophospholipases, not shown, have in some cases properties similar to phospholipase B and are known to attack the acyl ester at either position 1 or position 2 of the glycerol backbone. Furthermore, some of the phospholipases C and D do not hydrolyze phosphoglycerides but use sphingomyelinase as their substrate. These phospholipases C are also referred to as sphingomyelinases. The products of that reaction are phosphocholine plus ceramide.

The recommended nomenclature for the phospholipases follows the pattern of differentiating between the acyl hydrolases and the phosphodiesterases (*Enzyme Nomenclature,* Academic Press, New York–San Francisco–London, 1979):

Recommended name(s)	EC number
Phospholipase A_1	3.1.1.32
Phospholipase A_2	3.1.1.4
Phospholipase B (lysophospholipase)	3.1.1.5
Phospholipase C	3.1.4.3
Sphingomyelin phosphodiesterase (phospholipase C)	3.1.4.12
Phospholipase D	3.1.4.4
Lysophospholipase D (alkylglycerophosphoethanolamine phosphodiesterase)	3.1.4.39
Sphingomyelin phosphodiesterase D	3.1.4.41

In addition to the differences in substrate specificity, considerable differences exist between the molecular and catalytic properties, as would be expected. Within a given class of phospholipases, however, a remarkable degree of homology is found. This is particularly true of the phospholipases A_2 from venoms and the mammalian pancreas. Considerable information on this homology and the evolution of the enzymes is available, and it is covered in some detail in this book. A final determination of the extent of homology in a given class of enzymes will, in most cases, require much additional work.

Clearly, the functions of phospholipases are diverse. Basically, two general types of function can be considered, even though this consideration is somewhat too simplistic. First, there is the action of phospholipases in digestion, which exists at many levels. Digestion of extracellular phospholipids can serve as a mechanism by which bacteria can derive a source of phosphate, while digestion of dietary neutral lipid and phospholipid is dependent on the action of phospholipases in the intestine; this action therefore provides the essential fatty acids required for normal metabolism.

Regulatory phospholipases have low catalytic activity and are present in small quantities in cells relative to the digestive enzymes. In addition, they are under stringent regulation that limits their activity, thereby protecting the cell from membrane degradation and cytolysis. These phospholipases have come under intense investigation of late with the recognition that the formation of a number of bioactive lipids such as the eicosanoids (e.g., prostaglandins and leukotrienes) is dependent on the action of phospholipases. Likewise, the very fundamental acyl composition of the phosphoglycerides that constitute the bilayer of membranes is dependent on phospholipase action in a deacylation–reacylation cycle.

This book attempts to cover many aspects of the basic enzymology of phospholipases in addition to some functional aspects of physiologic action, especially in Chapter 11. While the book is primarily organized phylogenetically, there are some notable exceptions to this rule. This is more evident toward the later portion when mechanisms of action and function are covered. The first chapter is oriented toward the basic considerations of the nature of the substrate and methods of enzyme assay. In all cases, useful references to more detailed descriptions of the material are given so that the reader may pursue those areas of interest beyond the scope of this book.

Moseley Waite

Acknowledgments

Many people contributed to this book, people to whom the author is deeply indebted. A number of the concepts and conclusions reached here have been gleaned in some fashion from others, for there are a number of excellent scientists working in one fashion or another on phospholipases. However, in a book such as this, some inaccuracies, omissions, and overstatements exist; those shortcomings clearly are the responsibility of the author.

Specific acknowledgments for all those who responded to correspondence and provided data are indicated in the text; I am greatly appreciative of this help. I would like to give particular recognition to the following, who spent considerable time in discussion of phospholipases with me: Drs. H. van den Bosch, A. Slotboom, G. H. deHaas, G. Scherphof, A. Bangham, R. Verger, S. Gatt, P. Elsbach, R. Franson, M. Wells, W. Parce, M. Thomas, P. Cullis, K. Chepenik, B. Smith, R. Lumb, E. Dennis, R. Heinrikson, S. Jackowski, C. Rock, P. Majerus, R. Snyderman, G. Weissmann, M. Jain, and A. Abdel-Latif. Special thanks go to Dr. D. Hanahan, who conned me into writing this book but who paid the price by reading rough drafts; his comments, insights, and encouragements were invaluable.

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This book would not exist without the heroic efforts of Tommye Campbell. From hundreds of pages of handwritten manuscript and duplicate copies of figures and tables, she carved out the finished copy. Likewise, she handled permissions correspondence and kept references in order. I will not be able to show adequate appreciation other than to simply say thanks and it's done.

Much of the initial writing was done in the Department of Biochemistry at the University of British Columbia; Drs. Jean and Dennis Vance were wonderful hosts in a magnificent environment.

M.W.

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Chapter 1

Assay of Phospholipases

The purpose of this chapter is to describe briefly some of the most commonly used assays for the different classes of phospholipases. Also, some less frequently used assays are described that potentially could be of use under certain conditions. In the following chapters, mention is made of the specifics of the assay used in a particular study and reference to Chapter 1 can be useful as a general reference. Some chapters focus on substrate–enzyme interaction and therefore give better insight into the molecular mechanism of the assays used; this is particularly true of Chapter 10.

1.1. General Considerations and Choice of Assay

It is of interest to note changes in the analytical methods used over a period of some 40 years and to imagine what the state of the field would be without the vast improvements in assays that have occurred. In her classical early studies on the toxin of *Clostridium welchii,* Macfarlane (Macfarlane and Knight, 1941) wrote the following: "A typical hydrolysis was carried out as follows: 830 mg lecithin, emulsified in H_2O, 1.5 ml 0.1 M $CaCl_2$ and 5 mg dry *Cl. welchii* toxin (phospholipase C) were mixed in a total volume of 15 ml adjusted to pH 7.4 with 0.1 N NaOH and placed in a thermostat at 37°; a further 2 mg toxin were added after 5 hr and the mixture was titrated back to pH 7.4 with 0.1 N NaOH at intervals until no further liberation of acid took place, by which time a layer of fat had risen to the surface." This was then assayed by chemical hydrolysis of the product phosphocholine followed by choline and phosphorus determinations. Furthermore, the phosphocholine was crystallized and elemental analysis performed. The neutral lipid (diacylglycerol) was assayed for its saponification value and the freed fatty acids separated into saturated and unsaturated fatty acids based on the solubility of the Pb^{2+} salts. While this rigorous approach was essential at that time for the characterization of the products of phospholipase action, it would hardly lend itself to the types of study ongoing today, such as enzyme purification, or to the action of phospholipases within membranes.

The assay of phospholipases is somewhat complex owing to the water-

insoluble nature of the substrate employed. While it has been shown that phospholipase A_2 can hydrolyze phosphatidylcholine in the monomeric form, optimal activity is reached only above the critical micellar concentration (CMC) (Roholt and Schlamowitz, 1961; Pieterson *et al.*, 1974). Because phospholipases preferentially hydrolyze substrate above their CMC, it is essential in the study of phospholipases to know the physical properties of the substrate. To simplify the understanding of the substrate, most investigators have employed model systems as substrates such as micelles or liposomes. Figure 1-1 illustrates three phases of lipid aggregates to be discussed as model substrates (Cullis and Hope, 1985). Even though these usually are not the physiological substrates, the properties of model membranes are simple relative to the complexities of the natural substrates, that is, biological membranes. As our understanding of phospholipase–model substrate interaction increases, undoubtedly more emphasis will be placed on the molecular mechanisms of phospholipase–membrane interaction. Indeed, some very elegant studies involving the activity of phospholipase–red cell membrane interaction have been carried out that have led to a better understanding of the molecular packing and architecture of phospholipids in this membrane.

The method of assay has depended on the type of phospholipase and its catalytic turnover rate. Many of the extracellular phospholipases (venom, bacterial, pancreatic) have specific activities higher than 1000 μmoles/min/mg.

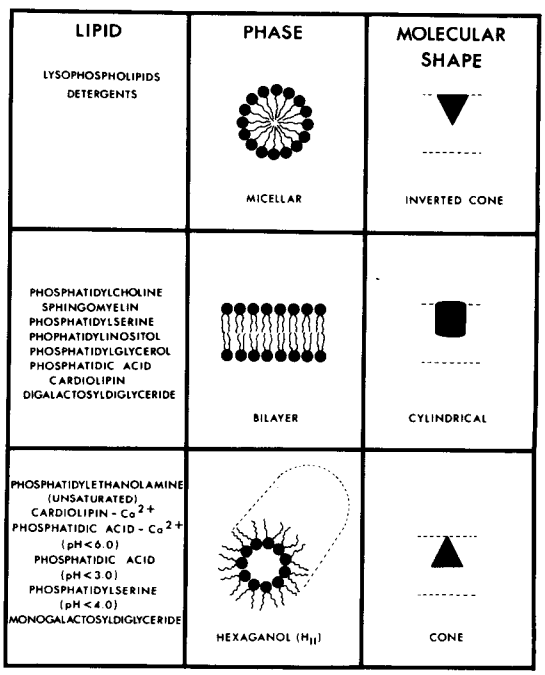

Figure 1-1. The polymorphic phases of phospholipids are cartooned in this figure. A major factor dictating the molecular shape and therefore the phase is the relative cross-sectional area of the polar head group and the hydrocarbon acyl chains. (From Cullis and Hope, 1985.)

It is quite convenient to assay these enzymes using a titration procedure measuring the proton liberated during hydrolysis. On the other hand, workers studying the less active cellular phospholipase often employ radio tracer techniques that are extremely sensitive. These techniques generally suffer from being much more laborious, however. Recently, Aarsman *et al.* (1976) have developed a sensitive colorimetric assay for phospholipases A using model compounds with thiol acyl esters that has some attractive features including sensitivity and simplicity. The product of the reaction contains a free sulfhydryl that can be coupled with 5,5′-dithiobis-2-nitrobenzoate (Elman's reagent). It is not certain at this point that these model compounds are suitable substrates for all phospholipases A_2, and the kinetic values obtained with these are usually different from those obtained with the natural oxy acyl ester substrates. Some less commonly used or limited types of assay that have been reported will not be covered even though for the particular problem under investigation they were quite well suited. These include charring of product on thin-layer chromatography plates or the interesting new variation of this using flame ionization detection of phospholipids and hydrolysis products separated on coated quartz rods (Ackman, 1981). Bioluminescent (Ulitzur and Heller, 1981), fluorimetric (Gatt *et al.*, 1981; Hendrickson and Rauk, 1981), and hemolytic (Vogel *et al.*, 1981) assays have been described that could become quite valuable in this area of research once their generality has been established. Likewise, gas chromatography or high-pressure liquid chromatography of fatty acids and glyceride products will not be covered. The procedures are described elsewhere in *Methods in Enzymology* (Patten *et al.*, 1981; Porter and Weenen, 1981) or in Kates' detailed chapter entitled "Techniques in Lipidology" (Kates, 1972).

1.1.1. Physical Form of Substrate

A major factor regulating phospholipase action is the initial interaction of the enzyme with bulk-phase substrate prior to the binding of the substrate molecule into the active site of the enzyme. For this reason, knowledge of the aggregated state of the substrate is essential. The following sections are brief descriptions of various forms of substrate used for phospholipase assays, lipid preparations, and methods of assay. For more details of phospholipase–substrate interaction, the reader should refer to Chapters 9 and 10 and to Verger (1980), Verger and deHaas (1976), Dennis (1983), or Verheij *et al.* (1981*b*).

1.1.2. Liposomes

Undoubtedly, liposomes are the most commonly used substrates for measurement of phospholipase activity. While liposomes vary in complexity and size, they share a common property described by Bangham and coworkers over 20 years ago; namely, they are osmotically active bimolecular leaflets (Bangham *et al.*, 1965). In this form, the phospholipids are organized in a manner closely resembling biological membranes. There are two basic types

of liposome, ones with a single bimolecular leaflet and those with multilamellar leaflets. The size of the single-shell vesicles can range from approximately 25 nm (Huang and Lee, 1973) to 1.2 μm or greater (Kim and Martin, 1981); multilamellar vesicles can be even larger. Most methods of liposome preparation yield a range of vesicle sizes and, in some cases, a mixture of single and multilamellar vesicles. The characteristics of the liposome are dependent on the phospholipid composition, the solute composition, and, to a great extent, on the method of vesicle preparation. Certain pure phospholipids will not form single-shell vesicles; for example, phosphatidylethanolamine will not form vesicles unless it is mixed with nearly equal amounts of another phospholipid, usually phosphatidylcholine. Indeed, recent evidence obtained by nuclear magnetic resonance measurements indicates that phosphatidylethanolamine does not form the usual bilayer structure but rather forms a structure similar to hexagonal type II (Fig. 1-1; Cullis and deKruijff, 1978).

An extremely important question arises in the preparation of liposomes concerning the effective amount and concentration of phospholipid available to the enzyme at the lipid–water interface. This question exists under any conditions in which the substrate is not present in true solution. For example, phospholipid can be added to an aqueous solution such that the preparation has 1 μmole of phospholipid per milliliter of solution. While this concentration would be considered 1 mM, the effective concentration of the substrate on the surface of the liposome essentially would be pure phospholipid plus water of hydration. This would be on the order of 7.5–11.0 M assuming the approximate dimensions of 2.0 nm by 0.6 nm^2 for the phospholipid molecule in the bilayer (Cullis and Hope, 1985). The actual area is dependent on the acyl and polar head groups as well as the ionic composition of the medium. This consideration is significant since many, if not all, phospholipases bind to the liposome and effectively are active on substrate at this very high concentration. For this reason, classical Michaelis–Menten kinetics should not be employed as is done with soluble substrates. While there are many examples of K_m and V_{max} values being given for phospholipases, these serve only to demonstrate a relative affinity constant and maximal velocity under the conditions employed.

Figure 1-2 is a representation of the type of enzyme–liposome interaction that is thought to occur with many phospholipases, in particular, the A_2 type. This cartoon, drawn to the approximate scale of limited size single-lamellar vesicle and the porcine pancreatic phospholipase A_2 (2.2 × 3.0 × 4.2 nm) (Dijkstra *et al.*, 1978), has four rate factors that need consideration in addition to the catalytic rate of the phospholipase: (1) the rate at which the enzyme attaches to and leaves the liposome (on–off rate); (2) the rate at which the products of the reaction, monoacylphospholipid and fatty acid, diffuse from the enzyme in the outer monolayer; (3) the rate at which the product monoacylphospholipid and/or fatty acid "flip-flops" into the inner monolayer, possibly exchanging with a substrate diacylphospholipid; and (4) the rate at which the substrate diacylphospholipid diffuses to contact the phospholipase. Another possible factor that could influence the rate of hydrolysis is the exchange of phospholipid between particles, although this has been shown

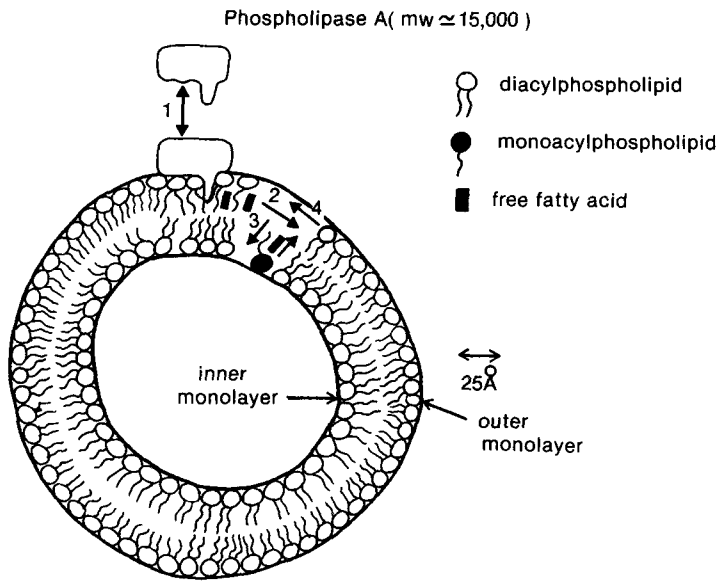

Figure 1-2. This cartoon shows the reversible binding of a phospholipase to a bilayer vesicle (step 1). Following hydrolysis, products (step 2) and substrate (step 4) diffuse away from the enzyme and bilayer "flip-flop" of lipid occurs (step 3).

to be extremely slow for micelles (Kornberg and McConnell, 1971). Two of the basic characteristics of the liposome that can influence the on–off rate of an enzyme are the electrokinetic surface properties (Bangham and Dawson, 1962) and the molecular packing of phospholipid molecules within the liposome (Hauser *et al.*, 1981). The motions of substrate, product, and enzyme in the liposome are interdependent events and combined, they could influence the overall observed rate of catalysis. This model does not take into account the activity of the enzyme of monomeric (soluble) molecules or the diffusion of product into the aqueous phase. Although these events can occur, probably they are negligible in magnitude. Indeed, Jain *et al.* (1980) and Hunt and Jones (1984) demonstrated that under conditions of limited hydrolysis, the bilayer structure is maintained. When the extent of hydrolysis exceeds one-half (outer monolayer) then the "flip-flop" of product and substrate can become a major factor in the limitation of activity. This becomes even a greater consideration when multilamellar vesicles are used as substrates. Potentially, the size of the vesicle becomes a consideration when one realizes that the total surface area ranges from nearly 2000 nm^2 to 4.5 μm^2 (diameter of liposome 25 nm to 1.2 μm). This 2200-fold difference in surface area could be of considerable importance in contrasting the relative contribution of the enzyme on–off rate (1) with diffusion rates (2–4). Also, the difference in surface area is related to the radius of curvature and hence the packing characteristics of the phospholipid molecules. A theoretical consideration of diffusion-controlled reactions in planar structures (two dimensions) has been developed by