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COX, JEFFREY WAYNE
SULFUR SUBSTITUTED ANALOGS OF PHOSPHOLIPIDS.
I. CHEMICAL SYNTHESIS OF SUBSTRATES FOR THE
SPECTROPHOTOMETRIC ASSAY OF PHOSPHOLIPASES.
II. STUDIES ON BACTERIAL PHOSPHOLIPASE C AND
RHIZOPUS DELEMAR LIPASE.

THE OHIO STATE UNIVERSITY, PH.D., 1979
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SPECTROPHOTOMETRIC ASSAY OF PHOSPHOLIPASES.
II. STUDIES ON BACTERIAL PHOSPHOLIPASE C
AND RHIZOPUS DELEMAR LIPASE.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Jeffrey Wayne Cox, B.S.

The Ohio State University
1979

Reading Committee:
Lloyd A. Horrocks, Ph.D.
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CHAPTER I

SUBSTRATES AND ASSAYS
FOR MAMMALIAN INTRACELLULAR
PHOSPHOLIPASES

Phospholipases catalyze the hydrolysis of ester linkages in phospholipid molecules (Figure 1). They seem to be ubiquitous in the plant and animal kingdoms (Brockerhoff and Jensen, 1974). The vast majority of experimental data on phospholipases concerns enzymes that are secreted into the culture medium of microorganisms, into toxic venoms, or into the digestive tracts of mammals (Brockerhoff and Jensen, 1974; Fu, 1978). Our knowledge of the physiological role of phospholipases in mammalian systems is constantly expanding, and it now appears that besides being required for the normal catabolism of phospholipids, phospholipases are mediators of various membrane responses to physiological stimuli (Hawthorne and Pickard, 1979; Michell, 1975; Michell et al., 1976; Horrobin, 1978). Excesses of phospholipase activity have been postulated to mediate cell injury during myocardial infarction (Franson et al., 1978a) and demyelinating diseases (Horrocks et al., 1978; D'Amato, 1974).
Figure 1. Sites of action of the various phospholipases
Rapid progress in mammalian phospholipase research is impeded by time consuming and tedious enzyme assay procedures. The low specific activity of phospholipases in mammalian tissues requires the use of radioactive substrates for enzyme assays. After incubation of the substrate with the enzyme preparation, the products are fractionated by extraction and chromatography. The assay system is complex since the lipases are oftentimes membrane bound and the substrates are in the form of plurimolecular aggregates. Furthermore, most methods to produce radioactive substrates yield a mixture of molecular species varying in chain length and degree of unsaturation. Recent advances in phospholipid chemistry have made chemically defined radioactive phospholipids more accessible to biochemists. In this brief survey, commonly used methods for radioactive substrate preparation are compared and newer synthetic approaches evaluated as possible alternatives. The feasibility of using sulfur substituted analogs of phospholipids as colorimetric substrates for phospholipases, which is the topic of this dissertation, will then be considered.

There are two categories of methods for the preparation of radiolabeled phospholipids, each of which can be subdivided into two classes. Biosynthetic methods consist of in vivo and in vitro labeling procedures whereby whole animals or semi-purified enzymes, respectively, are used
to incorporate a radioactive precursor into phospholipids. Chemical methods consist of either total or partial synthesis of a phospholipid molecule. A total synthesis begins with glycerol or optically active glycerol derivatives obtained from D or L-mannitol, whilst a partial synthesis utilizes the glycerol moiety of an existing phospholipid molecule. In the past, biosynthetic methods have been used almost exclusively to prepare labeled phospholipids because of their simplicity. Unfortunately, a wide variety of molecular species with varying specific radioactivity is usually obtained.

For example, intraportal injection of (Me-^3H)choline into rats results in preferential labeling of 1-acyl-2-linoleoylglycerophosphocholine (GPC) over 1-acyl-2-arachidonoyl or 1-acyl-2-docosahexaenoylGPC in liver lipids (Salerno and Beeler, 1973). On the other hand, injection of (^3H)ethanolamine preferentially labels 1-acyl-2-docosahexaenoylGPE (Salerno and Beeler, 1973; Sundler, 1973). The mono and dienoic fractions of rat liver diacylGPC are preferentially labeled by injection of 2-(^3H)glycerol (Kanoh, 1970; Åkesson et al., 1970). (^14C)Palmitate (as the BSA complex) injected intraportally into rats was mainly incorporated into the 1-acyl group of liver diacylGPC and GPE (glycerophosphoethanolamine), with most of the label in the 1-palmitoyl-2-arachidonoyl species (Åkesson et al., 1970b). As long as these
experiments are terminated after maximal incorporation of the label into the desired phospholipid, the majority of the radioactivity will be found in the expected moiety. With the exception of $^{32}\text{P}_i$ incorporation into phospholipids (Scandella and Kornberg, 1971; Mauco et al., 1979; Gatt et al., 1966; Colbeau et al., 1977), the labeled products isolated from these experiments must be subjected to chemical or enzymic degradation to establish the location of the radioactivity. Unless the phospholipid is further subfractionated into unsaturated species by AgNO$_3$ chromatography (Arvidson, 1965; Renkonen, 1966), and this has never been done for phospholipase studies, the results of phospholipase assays may be misleading because the specific radioactivity of the individual molecular species varies. Experiments relying on dual isotope analyses to determine the type of phospholipase activity must be interpreted with caution when both labeled substrates have been prepared by biosynthetic methods. For example, a change in the isotope ratio of $^{32}\text{P}$ and 1-(14C)acyl labeled phospholipid compared to the lyso product may indicate the preferential hydrolysis of the 1-ester group by a phospholipase A$_1$ or it may result from the preferential hydrolysis of the $^{32}\text{P}$ labeled substrate relative to the 14C substrate by a phospholipase A$_2$. Gatt et al. (1966) and Waite and van Deenen (1967) ignored this possibility.
Other in vivo methods utilize microorganisms to incorporate $^{32}\text{P}_i$, $(^{14}\text{C})$glucose, $(^{14}\text{C})$glycerol, or $(^{14}\text{C})$fatty acids into the desired lipid (reviewed by Kates, 1972). The radioactive precursor is simply added to the growth medium and the cells harvested after an appropriate time interval. In at least one case the cell membranes are used directly without purification of the labeled phospholipid substrate. Franson et al. routinely prepare phospholipase A substrate by growing E. coli in the presence of $(^{14}\text{C})$oleate followed by autoclaving the cells to render the membranes more susceptible to hydrolysis (Franson et al., 1978; Franson and Waite, 1978; Franson et al., 1974). About 95% of the incorporated label is in phospholipid, of which 50-65% is associated with PE. Of the $(^{14}\text{C})$oleate in phospholipid, about 95% is in the 2-position. A related approach utilizing in vivo biosynthetic methods is to assay phospholipases with endogenously labeled substrates. Platelet phospholipase A$_2$ has been measured in situ with endogenous $(^{14}\text{C})$phospholipid generated by incubation of the platelets with $(^{14}\text{C})$arachidonic acid (Rittenhouse-Simmons and Deykins, 1978). Assays were started by the addition of Ca$^{2+}$ and measured the loss of label from one of the several lipid components separated by TLC.

With the above exceptions, most phospholipase A substrates are generated in vitro by the incubation of an
appropriate lyso phospholipid with labeled fatty acid, CoA, ATP, MgCl₂, and microsomal acyltransferase. Robertson and Lands (1962) first described this technique for the preparation of either 1 or 2-([¹⁴C])acyl 1,2-diacylGPC, and several modifications have been reported (Gatt et al., 1966; Waite and van Deenen, 1967; Waite and Sisson, 1973; Woelk and Porcellati, 1973; Sun et al., 1979). Depending on the homogeneity of the lyso phospholipid acceptor molecule, this method will produce mainly one molecular species of labeled phospholipid. However, the amount of exogenously added cold phospholipid already present in the microsomal membranes frequently exceeds the mass of the lyso phospholipid used in the incubation. The result is the equivalent of adding a carrier phospholipid which is not identical to the labeled material. On the other hand, in vitro techniques utilizing phospholipid free enzymes can be used quite conveniently to obtain homogenous radio-labeled phospholipids. This approach has only been recently developed and is more appropriately discussed in context with chemical synthesis procedures.

Several excellent review articles have chronicled advances in phospholipid chemistry (Rosenthal, 1975; Paltauf, 1973; Slotboom and Bonsen, 1970; Jensen and Pitas, 1976; van Deenen and de Haas, 1964). Of interest to phospholipase investigators are the recent developments in the partial synthesis of phospholipids. For example,
sn-3-GPC may be prepared from egg or soybean diacylGPC by alkaline hydrolysis, and subsequent reacylation provides a 1,2-diacyl-sn-glycero-3-phosphocholine molecule with two identical fatty acids (Slotboom and Bonsen, 1970). Aneja extended this procedure to the preparation of diacylGPE (Aneja et al., 1970). N-TritylGPE was prepared from commercial diacylGPE (presumably free of alkenylacyl and alkylacylGPE) by tritylation and deacylation. Since the fatty acid chloride gave a high percentage of side reactions, the reacylation of GPC and N-tritylGPE was accomplished with the fatty acid anhydride. The harsh conditions of 48 hours at 80°C required for the reacylation limited the synthesis to saturated or monounsaturated fatty acids. Gupta et al. (1977) found that if N,N-dimethyl-4-aminopyridine was used as the base for the acylation reaction with the anhydride, yields of 75-90% were obtained at room temperature with a reaction time of 30 hours. By the use of fatty acid imidazole as the acylating agent, Warner and Benson (1977) shortened the reaction time to 4 minutes at 17°C. The fatty acid imidazole can be prepared by reaction of fatty acid and carbonyldiimidazole at room temperature in tetrahydrofuran for 45 minutes. These workers reported a yield of 63% for the synthesis of 1,2-dilinolenoylGPC. Both procedures, involving N,N-dimethyl-4-aminopyridine as base or fatty acid imidazole as the acylating agent, can be used for the
preparation of 1,2-diacyl-(N-trityl)GPE or 1,2-diacyl-
(N-tert-butoxycarbonyl)GPE. The blocking groups can be
removed by treatment with acetic acid (or silicic acid)
or HCl, respectively (Slotboom and Bonsen, 1970;
Lammers and van Boom, 1977). These methods represent
the simplest route to 1,2-diacylGPC with identical fatty
acids. 1,2-DiacylGPE is more conveniently prepared by
transphosphatidylation of 1,2-diacylGPC with cabbage
phospholipase D as discussed later.

Mixed acid phospholipids can be obtained by treatment
of the monoacid diacylGPC or tert-butoxycarbonylGPE with
phospholipase A2 to produce the 1-acyl lysophospholipid
which can be reacylated by the same procedures described
above. It should also be possible to reacylate at posi­
tion 1 after hydrolysis with phospholipase A1, though
particular care must be taken to avoid isomerization of
the 2-acyl to the 1-acyl lysophospholipid (Robertson and

As in these methods for the acylation of GPE, a
blocking and deblocking step must be added on account of
the amine group. This is a general problem for all
phospholipids with bifunctional polar head groups. As a
result, PC is generally easier to prepare and obtainable
in higher yields. It was therefore an important dis­
covery by Benson et al. (1965) that cabbage phospholipase
D catalyzes the transphosphatidylation of the phosphatidyl
moiety of PC to alcohol receptor groups other than water (Figure 2). This was followed by several studies on the preparative use of the reaction (reviewed by Yang and Morrisett, 1977). Phospholipase D exchange reactions can be used to prepare radiolabeled phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, and phosphatidyl serine by exchange of cold phospholipid with the appropriate radioactive receptor alcohol (Yang and Morrisett, 1977). Spin labeled PC can be prepared by exchange with TEMPO-choline (Yang and Morrisett, 1977). Saito et al. (1974) demonstrated that whereas choline, ethanolamine, glycerol, and serine were active receptor alcohols, inositol, threonine, glucose, and glycerophosphate were not. Kovatchev and Eibl (1978) examined the steric requirements of savoy cabbage phospholipase D for the receptor alcohol, and found that alcohols of up to six carbon chain length were acceptable. Substitution of carbon atoms with atoms possessing covalent radii greater than 1.0 Å produced unacceptable alcohols. Thus, primary alcohols up to hexanol, alkanediols up to 1,5-pentanediol, allyl and propargyl alcohol, 1-fluoro or 1-chloro-2-ethanol, ethanolamine, and propanolamine all gave yields of better than 50% for the exchange reaction. This method represents the simplest route to many of the phospholipids containing bifunctional alcoholic groups.
Figure 2. Schematic illustration of the phospholipase D exchange reaction (from Yang and Morrisett, 1977). Phospholipase D (PLD) is a sulfhydryl enzyme (HS-E).
Total or de novo synthesis routes for these types of phospholipids have also been improved. Lammers and van Boom (1977) recently described the use of a phosphotriester intermediate to prepare phosphatidyl ethanolamine and phosphatidyl glycerol (Figure 3). A 1,2-diglyceride is first reacted with a monofunctional reagent, R'R"POCl, where R' is trichloroethanol and R" is aniline. The aniline group of the phosphotriester intermediate (I) is selectively removed by treating with isopentyl nitrite in pyridine/acetic acid and the alcohol group is then attached to the phosphodiester intermediate (II) by the use of a condensing reagent such as 2,4,6-triisopropylbenzenesulfonfyl chloride. Trichloroethanol is then removed with p-toluenesulfonic acid/pyridine/Zn dust to afford (III). Finally, the trityl blocking group is removed to yield phosphatidyl ethanolamine (IV). This is a complicated synthesis requiring four column chromatography steps, but the low incidence of side reactions allows an overall yield of 70%.

In contrast, a route to phosphatidyl ethanolamine that is comparable in simplicity to the classical phosphatidyl choline synthesis of Hirt and Berchtold (1958) has been developed by Eibl (1978) (Figure 4). Reaction of the 1,2-diglyceride with phosphorus oxytrichloride yields the dichlorophosphatidate, which can then be reacted directly with ethanolamine to yield an oxazaphospholane (V). The
Figure 3. Synthetic scheme for PE and PG using phosphotriester intermediates according to Lammers and van Boom (1977).
Figure 4. Synthetic scheme for PE using an oxazaphospholane intermediate (V) according to Eibl (1978). R is 1,2-diacylglycerol.

Figure 5. Synthetic scheme for cardiolipin using cyclic enediol phosphorylating reagents according to Ramirez and Maracek (1978).
ring is opened under acidic conditions to provide phosphatidyl ethanolamine in 90% yield. This route is particularly attractive in that no workup is required until the synthesis is complete.

The most general route to the synthesis of phospholipids yet described has been developed by Ramirez and co-workers (summarized by Ramirez and Maracek, 1978) (Figure 5). Reaction of a diglyceride with cyclic enediolpyrophosphate (VI) yields a cyclic triester still containing the enediol functionality (VII). A second alcohol opens the enediolphosphate ring to provide a dialkyl 3-oxo-2-butylphosphate (VIII). The α-ketol group is then hydrolyzed in aqueous pyridine containing triethylamine. This procedure has been used to synthesize cardiolipin (40% overall yield), cholesteryl phosphocholine, and phosphatidyl choline. Other bifunctional alcoholic groups could also be used as long as they were suitably protected. This method, like that of Eibl (1978) for phosphatidyl ethanolamine synthesis, has the advantage that it requires only workup of the final product.

Racemic diglycerides for these syntheses can be prepared by partial lipolysis of triglycerides or by a four step synthesis from allyl alcohol (Jensen and Pitas, 1976). It is considerably more difficult to obtain 1,2-diacyl-sn-glycerols from D or L-mannitol (Mattson and Volpenhein, 1960; Chacko and Hanahan, 1968). However, these may be
rather simply obtained by partial hydrolysis of racemic lecithins by *Bacillus cereus* phospholipase C (see Chapter III of this dissertation).

The commercial availability of PC with identical fatty acids should make partial synthesis routes particularly attractive to most biochemical laboratories (Figure 6). Labeled fatty acids can be incorporated by deacylation-reacylation reactions employing phospholipases to specifically remove one fatty acid. Commercial *Crotalus adamanteus* phospholipase A$_2$ and *Rhizopus delemar* lipase (a phospholipase A$_1$; see Chapter IV and V of this dissertation) are advised for positionally specific hydrolysis. The alcohol moiety of the polar head group can then be exchanged with phospholipase D to provide PE, PG, or PS. If 1,2-diacyl-sn-GPC is not available or is too expensive for use as starting material, an inexpensive racemic lecithin may be used for the preparation of PC labeled at the 2 position and for either of the labeled substrates for PE, PG, and PS. This is because the phospholipase A$_2$ and phospholipase D are stereospecific and will resolve the racemate (Batrakou et al., 1975; van Deenen and de Haas, 1966). On the other hand, *R. delemar* lipase is not stereospecific and starting with a racemate will yield a racemic lecithin labeled at the 1 position. PC, PE, PG, and PS can also be labeled in the polar head group by phospholipase D catalyzed exchange with the appropriate alcohol. An exchange reaction
Figure 6. Synthetic scheme for the preparation of radiolabeled phospholipids.
for the 1-acyl group of 1,2-diacylGPE has been reported by Brockerhoff et al. (1976). Incubation of labeled fatty acid and PE with _R. delemar_ lipase at pH 3.4 results in incorporation of about 20% of the label into the 1 position but only about 20% of the starting PE was reisolated. The rest was hydrolyzed to lysoPE. The exchange worked poorly with PC and not at all with PS or PI.

Thus, a single molecular species of the major types of phospholipids can be prepared relatively simply by a combination of chemical and enzymic procedures. These methods can also be used to incorporate the desired radioactive label and should supplant existing _in vivo_ and _in vitro_ biosynthetic methods.

Although radiochemical assays for phospholipases provide the necessary degree of sensitivity for mammalian systems, they are time consuming and cumbersome for routine work. Our knowledge of phospholipases has reached the point where the enzymes can be partially purified and studied for control mechanisms with various physiological and pharmacological modulators. Aarsman et al. (1976) have suggested that thioester analogs of phospholipids might be useful for the assay of lipolytic enzymes. The principle of the assay, which was first developed for acetylcholinesterase by Ellman et al. (1961), is that thioester hydrolysis will release a sulfhydryl group that can then react with colorimetric thiol reagents such as
5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (Figure 7). Unlike other spectrophotometric assays of esterases, the structure of the artificial substrate can be made identical to the natural substrate with the exception of sulfur substitution; the structural modification is minor. Aarsman et al. (1976) assayed porcine pancreatic phospholipase A\textsubscript{2} with a glycol lecithin prepared from mercaptoethanol. This substrate was known to satisfy the minimum structural requirements of the enzyme. It was also micellar, which meant a large surface area with low turbidity, and the product thiol was water soluble.

Because the glycol lecithin substrate is not a strict structural analog of a glycerophospholipid, however, its use is limited to certain well characterized systems. For example, \textit{Bacillus cereus} phospholipase C is known to hydrolyze 2-acylGPC but not 1-acylGPC, even though the locus of enzyme action is the glycerophosphoester linkage (de Haas and van Deenen, 1965). It follows that some phospholipases A\textsubscript{2} may have binding sites for the 1-acyl group. Provided that the glycol lecithin is an active substrate for a phospholipase A\textsubscript{2}, a specific enzyme assay requires selective inhibition of lysophospholipase activity (Aarsmen and van den Bosch, 1977).

On the other hand, the use of strict structural analogs of glycerophospholipids could introduce several problems. Phospholipids with long chain fatty acids usually produce
Figure 7. Thioester substitution assay for acetylcholinesterase using DTNB.

\[
\text{CH}_3\text{C-S-CH}_2\text{CH}_2\text{N(CH}_3\text{)}_3^+ \xrightarrow{\text{AChE}} \text{CH}_3\text{CO}_2\text{H} + \text{HS-CH}_2\text{CH}_2\text{N(CH}_3\text{)}_3^+ 
\]

\[
\text{O}_2\text{N-} \quad \text{S-S-} \quad \text{NO}_2 \xrightarrow{\text{RSH}} \quad \text{O}_2\text{N-} \quad \text{S-} \quad \text{NO}_2 
\]

\[\varepsilon(412\text{nm}) = 1.28 \times 10^4\]
an emulsion with a high degree of turbidity that could interfere with spectrophotometric assays (Aarsman and van den Bosch, 1977; Ottolenghi, 1963). The limited water solubility of the thiol from phospholipase A₁, A₂, C, and plasmalogenase hydrolysis of appropriate sulfur substituted analogs may affect its reactivity with the water soluble thiol reagent. Problems unrelated to the structure of the substrate, but which pose serious threats to the successful application of sulfur-substrate assays, are that some phospholipases are inhibited by DTNB (Aarsman and van den Bosch, 1977; Snyder et al., unpublished), that the pH range over which DTNB gives theoretical values for SH content is only 7.0-8.5 (Grassetti and Murray, 1967), and that thio linkages may be less susceptible to hydrolysis than the corresponding oxy linkage.

Considering each problem separately, the turbidity of phospholipid emulsions can be minimized by using intermediate chain length fatty acids. This may reduce the affinity of the substrate for enzymes with specific fatty acid requirements, but the remaining functional groups of the glycerophospholipid are intact and, with fatty acids longer than C₉, the critical micelle concentration is still very low (Bonsen et al., 1972). Regarding DTNB enzyme inhibition, Snyder et al. (unpublished) observed that inhibition of the brain lysophospholipase by DTNB was dependent on the reagent concentration. An acceptably
linear progress curve for the hydrolysis of Aarsman's glycol lecithin substrate could be obtained if the DTNB concentration was reduced from 1 mM to 0.2 mM. Enzyme inhibition may also be dependent on the charge of the SH reagent, and a suitable alternative to DTNB is 4,4'-dithio pyridine (Grassetti and Murray, 1967). This compound can be used under acidic conditions, since it has been shown to give theoretical values for SH content from pH 3.5-8.0 (Grassetti and Murray, 1967). Finally, although thioesters are reportedly hydrolyzed faster than oxyesters, there are no reports on the relative rates of hydrolysis of thio-phosphoester (phospholipase C) or vinyl sulfide (plasmalogenase) bonds. Chemically, thiophosphoesters are much more susceptible to acid catalyzed hydrolysis than the oxyphosphoesters (Bruice and Benkovic, 1966), while the converse is true of vinyl sulfides and vinyl ethers (McClelland, 1977).

Despite the potential problems, this assay technique is still the most promising candidate to replace radio-isotope assays for enzyme purification, kinetic analysis, and drug structure activity relationship experiments. The practical sensitivity limit of DTNB/SH assays approaches the low levels of phospholipase activity found in crude homogenates: 10 nmol/h corresponds to an absorbance change of 0.16/h. It is the purpose of this dissertation to develop spectrophotometric assays for phospholipases
A₁, C, and plasmalogenase using sulfur substituted phospholipids. An attempt is made to provide the substrates in the highest possible purity by the most convenient synthetic procedures. Microbial phospholipases are used as enzyme sources to develop the phospholipase C and A₁ assays, and some of their properties are examined.
CHAPTER II
THE SYNTHESIS OF CHOLINE AND ETHANOLAMINE PHOSPHOLIPIDS
WITH THIOPHOSPHOESTER BONDS:
POTENTIAL SUBSTRATES FOR PHOSPHOLIPASE C

Introduction

Phospholipase C (EC 3.1.4.3, phospatidyl choline cholinephosphohydrolase) catalyzes the hydrolysis of the glycerophosphoester linkage of phospholipids. Although the phosphatidyl choline hydrolytic activity can be assayed by a variety of methods (Ottolenghi, 1969), the only sensitive, continuous, spectrophotometric assay measures the release of p-nitrophenol from the artificial substrate, p-nitrophenylphosphocholine (Kurioka and Matsuda, 1976). Unfortunately, this chromogenic substrate differs markedly in physical and structural properties from glycerophospholipids, and absolute specificity for measurement of phospholipase C in heterogenous enzyme mixtures is uncertain.

A more subtle structural modification of glycerophospholipids that might allow a specific, spectrophotometric assay is the substitution of sulfur for oxygen in the glycerophosphoester linkage. Ellman first used this
approach to assay acetylcholinesterase with acetylthiocholine (Ellman et al., 1961). Compared to oxyphosphoesters, thiophosphoesters have similar alkaline stability but are more acid labile (Bruice and Benkovic, 1966). Hydrolysis of thio analogues of glycerophosphoesters by phospholipase C would produce water-insoluble mercaptoglycerides that may be reactive with colorimetric sulphydryl reagents. Of the lipolytic enzymes, lysophospholipase, phospholipase A$_2$ and triglyceride lipase have been assayed with thioester substrates, and all reactions yielded water soluble thiols (Aarsman et al., 1976; Aarsman and van den Bosch, 1977).

From a practical standpoint, the most dependable thiophosphoester substrates for phospholipase C should be easily dispersible to avoid high turbidity, yet mimic the physical state of naturally occurring phospholipids in aqueous systems. Egg yolk and soybean phosphatidyl cholines form bilayered vesicles upon ultrasonic irradiation in an excess of water (Tanford, 1973). The shortest chain, and most easily dispersible, homologue that behaves similarly is dinonanoylGPC (Tausk et al., 1974). The phospholipase C from both \textit{Clostridium perfringens} and \textit{Bacillus cereus} preferentially hydrolyze medium chain phosphatidyl cholines (Roberts et al., 1978, van Deenen et al., 1961). Thus, a thiophosphoester analogue of
didecanoylGPC should satisfy both structural and physical properties for a specific phospholipase C substrate.

An alternative substrate with a long chain fatty acid is the thiophosphoester analogue of palmitoylglycolphosphocholine. The oxy form of this lipid was prepared by Bonsen et al. (1972) as a lysolecithin analogue. It has a critical micelle concentration of about 40 µM in 0.1 M NaCl, and the micelles are probably spherical (Bonsen et al., 1972). We report here the synthesis of three new phospholipids as potential spectrophotometric substrates for phospholipase C: \textit{rac-1-S-phosphocholine-2,3-O-didecanoyl-1-mercapto-2,3-propanediol} (XIIIa), \textit{1-S-phosphocholine-2-O-hexadecanoyl-1-mercapto-2-ethanol} (XIIIb), and \textit{1-S-phosphoethanolamine-2,3-O-didecanoyl-1-mercapto-2,3-propanediol} (XIV).
Materials and Methods

Infrared spectra were obtained with a Beckman Model 4230 Infrared Spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, Tennessee) after drying in vacuo over P₂O₅ for 24 hours. Analytical thin-layer chromatography was done with 0.5 mm layers of silica gel G (EM Laboratories, Elmsford, New York) prepared from a slurry of 35 g gel and 67 ml water. Spots were visualized with either I₂ vapor, phosphate spray reagent (Vaskovsky and Kostetsky, 1963), Dragendorf stain (Beiss, 1964), ninhydrin spray (Waldi, 1965), or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1958) (5 mM DTNB in EtOH-water, 2:1, pH 7). Pyridine and triethylamine were distilled from BaO, and chloroform was washed with water, dried with Na₂SO₄, and distilled from CaSO₄. Bromoethylphosphorusoxydichloride was prepared according to Hirt and Berchtold (1957) for the synthesis of β-phthalimidoethylphosphorusoxydichloride. Bromoethanol was added to a 3 mole excess of POC₁₃ in benzene, and the solution refluxed for 2.5 hours. It was then rotary evaporated at 55° C and the product vacuum distilled (70° C at 0.5 mm Hg) in 80% yield. **rac-1,2-Didecanoyl glycerol** was synthesized as described by Jensen and Pitas (1976) using boric acid to remove the dihydropyran blocking group (Gigg and Gigg, 1967). This was then converted into **rac-1,2-didecanoylGPC** according to Hirt and Berchtold.
Mercaptoglycerol was purchased from Evans Chemetics (Darien, Connecticut) and distilled, and mercaptoethanol was purchased from Eastman (Rochester, New York). Phospholipase D was prepared from green head cabbage (purchased from a local market) as described by Kovatchev and Eibl (1978). Phospholipase A₂ (Crotalus adamanteus, Sigma, St. Louis, Missouri) incubations were performed in ether as described by Wells and Hanahan (1969).

Preparation of rac-1,1'-dithiobis(2,3-propanediol) (Xa) and 1,1'-dithiobis(2-ethanol) (Xb). Mercaptoglycerol (IXa) or mercaptoethanol (IXb) (10 ml) was dissolved in 20 ml water and the pH adjusted to 9 with ammonium hydroxide. A few crystals of FeCl₃ were added and air was bubbled through the mauve mixture with vigorous stirring. The oxidation was monitored by measuring the disappearance of sulfhydryl groups with DTNB. When the concentration of sulfhydryl groups stabilized, the mixture was filtered and the residue washed with small portions of water. More FeCl₃ was added to the filtrate and the bubbling was resumed. This process was repeated until no sulfhydryl groups could be detected. (If the filtrate was still opaque, bubbling was continued until filtration gave a clear to light brown solution.) Rotary evaporation at 50°C with several additions of benzene was followed by drying overnight at 1 mm Hg over P₂O₅. Yield was 70 to 80% based on weight, and the products were not characterized.
Preparation of rac-1,1'-dithiobis (2,3-O-didecanoyl-2,3-propanediol) (XIa) and 1,1'-dithiobis (2-O-hexadecanoyl-2-ethanol) (Xlb). Mercaptoglycerol disulfide (Xa) (30 mmoles) was dissolved in 180 mmoles pyridine and 100 ml chloroform. Decanoyl chloride (150 mmoles) was added to this solution with rapid stirring. After cooling, the mixture was diluted with 200 ml chloroform and the stoppered flask was allowed to stand in the dark for three days. For the mercaptoethanol disulfide (Xb), 30 mmoles were dissolved in 90 mmoles pyridine and 100 ml chloroform, and 75 mmoles palmitoyl chloride was added. The mixture was then diluted and allowed to stand for 24 hours. After the reaction was complete, 50 ml water was added and the mixture stirred for 1 hour and then poured into 1.2 l of hexane and extracted with 500 ml water. The organic phase was filtered through silicone-treated phase separating paper (Whatman #1 PS) and rotary evaporated. The residue was dissolved in chloroform and passed through a 250 g column of basic alumina prepared in chloroform. Both products (XIa,b) were not retained by the column and eluted well ahead of the fatty acids in 75% yield. Compound (XIa) is an oil, whereas (Xlb) is a solid crystallized from EtOH-water. TLC (hexane-diethyl ether-acetic acid, 90:10:1) $R_f=0.20$ (XIa), and 0.40 (Xlb). The infrared spectra of both compounds are nearly identical((XIa), neat between NaCl plates; (Xlb),KBr disc) and exhibit the following
strong bands: 2920-2960, 2860, 1460, 1375 (C-H); 1740, 1150-1200 (ester); 1105 (secondary ester, absent in Xlb); and 1050 cm\(^{-1}\) (primary ester).

Preparation of rac-2,3-O-dedecanoyl-1-mercapto-2,3-propanediol (XIIa) and 2-0-hexadecanoyl-1-mercapto-2-ethanol (XIIb). (XIa) or (XIIb) (7 mmoles) were dissolved in 150 ml EtOH or EtOH-hexane (9:1), respectively. Dithiothreitol (14 mmoles) was added and the solution made alkaline with several drops of ammonium hydroxide (pH 9.6 with combination electrode). After standing for 90 minutes, the solutions were rotary evaporated at 30\(^{\circ}\) C and the residues dissolved in 100 ml hexane. These solutions were washed 4 times with 25 ml portions of water, filtered through phase separating paper, dried with Na\(_2\)SO\(_4\) and CaSO\(_4\), and rotary evaporated to yield the products (XIIa,b) in 100% yield by weight. No disulfide was observable by TLC. TLC (hexane-diethyl ether-acetic acid, 90:10:1) \(R_f=0.40\) (XIIa), and 0.60 (XIIb). The products (XII) migrate ahead of the starting materials (XI) in this system and stain positively for sulfhydryl groups with DTNB spray reagent. No spots could be detected with I\(_2\) near the origin where the 1,3-isomers would migrate. The infrared spectra of (XII) are identical to (XI), with the exception of a sharp moderate band at 2570 cm\(^{-1}\) for sulfhydryl group in (XII). No band was observed at 1690-1700 cm\(^{-1}\) where thioesters absorb strongly.
Preparation of rac-1-S-phosphocholine-2,3-0-didecanoyl-1-mercapto-2,3-propanediol (XIIIa) and 1-S-phosphocholine-2-0-hexadecanoyl-1-mercapto-2-ethanol (XIIIb). Compound (XII) (7 mmoles) was dissolved in 25 ml chloroform and added dropwise to a well stirred solution of 28 mmoles of bromoethylphosphorosyldichloride and 60 mmoles of triethylamine in 25 ml chloroform at 0-5°C. Hydrolysis, trimethyamination, and purification were performed as described by Aarsman et al. (1976). The trimethylamination residue was dissolved in chloroform-methanol-water (4:4:1) and stirred for 30 minutes with 50 g mixed ion-exchange resin (Analytical Grade Ag 501-x8, Bio-Rad Laboratories, Richmond, California). After filtration through phase separating paper, the solutions were rotary evaporated with several additions of benzene. Crude products were taken up in chloroform and eluted from 100 g columns of Unisil silicic acid with the following solvents: 1.0 l chloroform, 1.0 l chloroform-methanol (4:1), and 2.0 l chloroform-methanol (1:4). The phosphatidyl choline analogues (XIII) eluted in the third fraction. On thin layer chromatography, (XIIIa) migrated between didecanoyl-GPC and egg phosphatidyl choline, and (XIIIb) migrated between egg lysophosphatidyl choline and didecanoylGPC. Both compounds stained positively for phosphate and choline. TLC (chloroform-methanol-water, 65:35:4) \( R_f = \) 0.37 (XIIIa), and 0.28 (XIIIb). Yields for overall
(XII) to (XIII) conversion are 35%. Infrared spectra of (XIII) and didecanoylGPC are compared in Figure 9. Assignments are as follows: 3300 (bound water); 2970, 2940, 2860 (C-H); 1740, 1170 (ester); 1260 (symmetric PO$_2^-$); 1080 in XIIIa, 1095 in didecanoylGPE (asymmetric PO$_2^-$); 1060 ((P)OC, shoulder in XIIIa, strong in didecanoylGPC); 970, 920 (quaternary trimethyl ammonium salt); 870 (PO(C)); 820, 720-790 (O-P-O, absent in XIIIa). A single elemental analysis yielded the following results:

Calcd for (XIIIa, monohydrate)
(C$_{28}$H$_{58}$O$_8$NPS):C 56.07,H 9.75,N 2.34,P 5.17,S 5.34
found:C 55.89,H 9.90,N 2.33,P 5.03,S 5.10

Calcd for (XIIIb, monohydrate)
(C$_{23}$H$_{50}$)$_6$NPS):C 55.28,H 10.08,N 2.80,P 6.20,S 6.41
found:C 56.48,H 10.26,N 2.71,P 6.31,S 6.43

Preparation of 1-S-phosphoethanolamine-2,3-0-didecanoyl-1-mercapto-2,3-propanediol sodium chloride salt (XIV).
Conversion of (XIIIa) to (XIV) was accomplished by transphosphatidylation with cabbage phospholipase D and ethanolamine as described by Kovatchev and Eibl (1978). Ethanolamine (12 g) was added to 15 ml 0.4 M sodium acetate-acetic acid buffer containing 0.16 M CaCl$_2$, and the pH was adjusted to 5.6 with approximately 15 ml 12 N HCl. Additions of (XIIIa) (1 mmole) in 30 ml diethyl ether and 20 ml of enzyme solution were made. The flask was tightly stoppered and gently shaken at room temperature for 24 hours. The reaction was terminated by rotary evaporating...
the ether and adding 6 ml of 0.5 M Na₄EDTA, pH 10.5. Chloroform-methanol (4:1, 144 ml) was added to extract the products which were eluted from a 100 g column of Unisil silicic acid. Column solvents: 1.01 CHCl₃, 0.61 CHCl₃-MeOH (10:1), and 1.01 CHCl₃-MeOH (4:1). The phosphatidyl ethanolamine analogue (XIV), which eluted in the third fraction, migrated identically with bovine heart ethanolamine glycerophospholipids and was obtained in 30% yield. It stained positively for both amine and phosphate. TLC (chloroform-methanol-water, 65:35:4) Rₛ=0.85. Phosphorus content by weight: calcd for C₂₅H₅₀O₇NPS NaCl 5.18%, found 5.13%. The infrared spectrum (Figure 9) is distinguished by bands at 2400-3300, 1630, 1550 (N-H); 1230 (PO₂⁻); 1070 (PO₂⁻) and ((P)OC); 1035, 890 (C-C-N); and by the absence of bands at 820 and 720-790 cm⁻¹ (O-P-O). After purification, the yield of (XIV) was 36%.
Figure 8. Synthetic scheme for phospholipid analogs containing thiophosphoester bonds.
Figure 9. Infrared spectra of rac-2,3-didecanoylglycerol-1-phosphocholine (DDGPC), rac-1-S-phosphocholine-2,3-O-didecanoyl-1-mercaptop-2,3-propanediol (XIIIa), and rac-1-S-phosphoethanolamine-2,3-O-didecanoyl-1-mercaptop-2,3-propanediol (XIV) obtained as 10% (W/V) solutions in chloroform against a blank cell containing chloroform.
Results and Discussion

Potential colorimetric substrates for phospholipase C were made from mercaptoglycerol and mercaptoethanol (Figure 8). A critical factor in the success of these syntheses was the selection of a sulfhydryl blocking group that can be removed under mild conditions. Systems employing acid or heat to deblock the sulfhydryl group to give intermediates (XII) will promote isomerization of (XII) to thioesters. The isomers might be separated due to the polarity difference of sulfhydryl and hydroxyl groups. Thus, it should be possible to obtain (XIIb) in low yields by partial acylation of mercaptoethanol followed by column chromatography. Compound (XIIa) presents a more difficult problem. Parham and DeLaitsch (1954) reported that when alcohols or thiols compete for limiting amounts of dihydropyran in the presence of acid, the mixed acetal predominates. Reaction of mercaptoglycerol with DHP and p-TSA gave several products. With these \( \beta \)-hydroxyl mercaptans, the 6-membered pyran ring probably opens to allow formation of a 5-membered heterocyclic mixed acetal.

Sulfhydryl groups can also be blocked by mild oxidation to disulfides. The desired thiol (XII) can be obtained by reduction of the acylated disulfide (XI). Mercaptoglycerol or mercaptoethanol was first oxidized by passing a stream of air through an aqueous alkaline solution containing \( \text{FeCl}_3 \) (Crawhall et al., 1956). After acylation and purification, the disulfides (XI) were
reduced under mild conditions in alkaline ethanol (pH 9.6) at room temperature with dithiothreitol (Cleland, 1964). Attempts to reduce the disulfide (XIa) with hydrazine hydrate (Katz and Schroeder, 1954) failed. The dithiothreitol reduction products (XII) migrated ahead of the disulfides (XI) in an acidic TLC system and stained positively for sulfhydryl groups with a DTNB spray reagent, whereas the disulfides (XI) did not. The infrared spectra of the thiols (XII) were identical to the spectra of the disulfides (XI) with the exception of a sharp sulfhydryl band at 2570 cm$^{-1}$. No isomerization of (XII) to thioesters was detected by either TLC or infrared spectroscopy. Intermediates (XII) were converted to phosphocholine derivatives (XIII) by the classical methods of Hirt and Berchtold (1958) as modified by Aarsman et al. (1976). Again, no thioester was detected in the products by infrared spectroscopy. Since thioesters absorb strongly at 1690-1700 cm$^{-1}$, a contamination of only a few percent should be clearly visible in the infrared spectrum. However, the infrared spectra do support the presence of a thiophosphoester (Figure 9). The sulfur atom has little effect on the PO$_2$-symmetric and asymmetric stretches, which appear at 1260 and 1080 cm$^{-1}$, respectively, in the phosphatidyl choline analogs (XIII), and at 1260 and 1095 cm$^{-1}$ in rac-1,2-didecanoylGPC (Corbridge, 1969).
However, the S-P stretches are beyond the low energy end of the spectrum and are not seen. Thus, bands associated with C-O-P vibrations in didecanoylGPC will either be weakened or nonexistent in the spectra of (XIII). For example, the (P)OC stretching vibration at 1060 cm$^{-1}$ is very strong in didecanoylGPC, but only a shoulder to the PO$_2^-$-asymmetric stretch in (XIII). Likewise, the PO(C) band at 870 cm$^{-1}$ is relatively stronger in didecanoylGPC than in (XIII). Finally, the O-P-O bands, assigned at 815 and 750 cm$^{-1}$ by Akutsu and Kyogoku (1975), are present in didecanoylGPC but completely absent in (XIII). Elemental analysis as well as the $R_f$ values and staining behavior with TLC were also consistent with the presumed structure of (XIII). The phosphatidyl choline analogue (XIIIa) migrates slightly ahead of didecanoylGPC on TLC, even though both were prepared and purified by the same procedures ($R_f=0.37$ for (XIIIa) compared to 0.30 for didecanoylGPC with chloroform-methanol-water, 65:35:4).

Phospholipase D transphosphatidylation was used to make the phosphatidyl ethanolamine analogue (XIV) from the phosphatidyl choline analogue (XIIIa). Savoy cabbage and brussel sprout phospholipase D have been reported to be partially or totally stereospecific for 3-sn-phospholipids (Batrakow et al., 1975; Davidson and Long, 1958; de Haas and van Deenen, 1965). Other types of cabbage have lower phospholipase D activity that may have different properties.
(Davidson and Long, 1958). For example, savoy cabbage phospholipase D hydrolyzes lysolecithin (Davidson and Long, 1958; Kovatchev and Eibl, 1978), whereas the plastid phospholipase D from another cabbage does not (Kates, 1956). Our phospholipase D was prepared from green cabbage and failed to convert the lysolecithin analogue (XIIIb) into the ethanolamine derivative. In order to determine the degree of resolution of the racemic lecithin analogue (XIIIa) by the cabbage enzyme, we incubated both (XIIIa) and (XIV) with *Crotalus adamanteus* phospholipase A<sub>2</sub>. This enzyme is totally stereospecific for 3-sn-phospholipids (Long and Penny, 1957; van Deenen and de Haas, 1966). In a three hour incubation with a large excess of enzyme, 52% of racemic (XIIIa) was hydrolyzed compared to 74% of (XIV). The racemic mixture was thus partially resolved to give a product (XIV) enriched in the naturally occurring stereoisomer.

Infrared analysis confirms substitution of amine for trimethylamine in the transphosphatidylation product (XIV) (Figure 9). The purification procedure of (XIV) yields the sodium chloride salt, which causes the PO<sub>2</sub><sup>-</sup> bands to be shifted to slightly lower energies of 1230 and 1070 cm<sup>-1</sup> relative to (XIII) at 1260 and 1080 cm<sup>-1</sup>. The asymmetric PO<sub>2</sub><sup>-</sup> stretch at 1070 cm<sup>-1</sup> overlaps the (P)OC band. Again, the phosphatidylethanolamine analogue migrates ahead of didecanoylGPE on TLC
(Rf=0.55 for XIV, compared to 0.40 for didecanoylGPE, chloroform-methanol-water, 65:25:4) even though both were prepared and purified by identical procedures. This difference apparently results from S substitution, since both thiophosphoester compounds (XIIIa and XIV) exhibit increased TLC mobility relative to their oxygen counterparts.

Thiophosphoester analogues of phosphatidyl choline and phosphatidyl ethanolamine have been prepared in moderate yields using this synthetic route. The products (XIII and XIV) are chromatographically pure and without significant contamination from thioester isomers that could serve as substrates for phospholipase A2 or lysophospholipase. These compounds have been used as substrates for the spectrophotometric assay of phospholipase C (Cox et al., 1979).
CHAPTER III
A CONTINUOUS SPECTROPHOTOMETRIC ASSAY FOR
PHOSPHOLIPASE C USING THIOPHOSPHOESTER SUBSTRATES

Introduction

Phospholipase C (EC 3.1.4.3, phosphatidyl choline
cholinephosphohydrolase) is commonly found in the culture
supernatant of bacteria (Ottolenghi, 1969; Brockerhoff and
Jensen, 1974). Until recently, however, the phosphatidyl
choline hydrolyzing variety of phospholipase C has not
been reported in mammals. There is indirect evidence for
the presence of a phospholipase C in erythrocytes (Allan
and Michell, 1977a; Allan and Michell, 1977b) and plate-
lets (Lapetina, 1979), and a phosphatidyl ethanolamine
hydrolyzing enzyme has been suspected in rat brain
(Williams, 1973). A sensitive and convenient enzyme
assay would facilitate phospholipase C research, since
current assay methods either lack sensitivity or are
cumberSome for routine work. The low levels of activity
expected for mammalian tissues (less than 10 nmoles/h)
require radiochemical assays that demand a large expense
in both time and money. Kurioka and Matsuda (1976) have
described a spectrophotometric assay with a water soluble
substrate, but its questionable specificity for phospholipase C and its low reactivity with \textit{C. perfringens} phospholipase C restricts its use to well characterized systems.

In a previous paper, we reported the synthesis of phospholipids containing thiophosphoester bonds as potential spectrophotometric substrates for phospholipase C (Cox et al., submitted). Hydrolysis of the thiophosphoester bond will produce thiols that may be reactive with colorimetric thiol reagents. This type of assay, involving thioester substitution for oxyesters, has not been described for a substrate that yields water insoluble thiols upon hydrolysis, nor has it been described as a method to assay phosphatases. Thus, it was uncertain as to whether the enzyme would hydrolyze the thiophosphoester bond, and if so, whether the resulting thiol would be reactive with water soluble thiol reagents.

We report here that these phospholipid analogs are hydrolyzed by both \textit{Clostridium perfringens} and \textit{Bacillus cereus} phospholipase C, and that the reaction may be monitored conveniently in a spectrophotometer with a sensitivity limit of 2 nmoles/h (0.04 A/h). The assay is examined in detail for accuracy and artifacts, and it is found to be a useful tool for kinetic and substrate specificity studies.
Materials and Methods

Enzymes. *Clostridium perfringens* phospholipase C was purchased from Sigma Chemical Company (St. Louis, Missouri). *Bacillus cereus* phospholipase C was supplied as a lyophilized powder following ammonium sulfate flotation (Ottolenghi, 1969) courtesy of Dr. Abramo Ottolenghi.

Substrates. Thiophosphoester substrates (Cox et al., submitted) are described in Figure 10. The D-isomer of MG(PC) was prepared by incubation of rac-MG(PC) with *Crotalus adamanteus* phospholipase A₂ as described by van Golde and van Deenen (1967). The remaining diacyl phospholipid was assumed to be the D-isomer and was isolated by chromatography on a silicic acid column.

Substrate Suspension. Substrates were suspended by evaporating organic solvent from the lipid, adding 0.1 M 3-((N-morpholino)propane sulfonic acid (MOPS) buffer, pH 7.0, and either vortexing or sonicating. Optically clear suspensions of ME(PC) were obtained by vortexing for 1 minute at room temperature. MG(PC) and MG(PE) substrates had to be sonicated to obtain suspensions with low turbidity. Sonications were performed with the vessel immersed in ice using a Bronwill Biosonick III Sonifier (Will Scientific, Rochester, New York) at 35% intensity equipped with a 3/4 inch probe. MG(PC) (2 mM) was suspended with 2x2 minute bursts, and MG(PE) (0.4 mM) with 5x2 minute bursts in a volume of 15 ml.
Figure 10. Structures and nomenclature of phospholipids with thiophosphoester bonds. MG(PC), rac-1-S-phosphocholine-2,3-O-didecanoyl-1-mercapto-2,3-propanediol; MG(PE), 1-S-phosphoethanolamine-2,3-O-didecanoyl-1-mercapto-2,3-propanediol; ME(PC), 1-S-phosphocholine-2-O-hexadecanoyl-1-mercapto-2-ethanol.
Enzyme Assays. Spectrophotometric assays measured the release of sulfhydryl groups as detected by their reaction with 4,4'-dithiopyridine (DTP) (Aldrich Chemical Company, Milwaukee, Wisconsin) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma). Incubations normally contain substrate (1 mM ME(PC) or MG(PC) and 0.2 mM MG(PE), 0.6 mM DTP or DTNB, and 0.1 M MOPS buffer, pH 7.0, in a total volume of 1 ml. *C. perfringens* incubations also contained CaCl$_2$. Assays were normally run by continuously monitoring the increase in absorbance at 324 nm(DTP) or 412 nm(DTNB). Rates were calculated using molar extinction coefficients of $1.98 \times 10^4$ for DTP (Grassetti and Murray, 1967) and $1.28 \times 10^4$ for DTNB (Aarsman et al, 1976). Discontinuous assays for released thiol were run in the absence of DTP. Reactions were inhibited with EDTA at a given time and DTP was added to quantitate the free thiol. Rates were also assayed by measuring the amount of water soluble phosphorus produced in a given time. Reactions were inhibited with EDTA and the reaction mixture extracted with 4 volumes of chloroform-methanol (2:1). Phosphorus content of the aqueous phase was determined by the method of Gottfried (1967).

Critical Micelle Concentrations. The critical micelle concentration (cmc) was determined by the spectral shift induced by the incorporation of Rhodamine 6G into
the hydrophobic core of the micelles as described by Bonsen et al. (1972) in the presence of 0.1 M MOPS buffer at pH 7.0.
Results

Clostridium perfringens phospholipase C. Incubation of C. perfringens phospholipase C with lysolecithin analog ME(PC) in the presence of DTP results in an increase in absorbance at 324 nm with time (Figure 11). The slope of the recorder tracing is dependent on the substrate concentration. The incubations were also monitored at 450 nm, where neither DTP nor thiopyridone absorbs, as an indication of changes in turbidity. During the initial stage of hydrolysis there was no change in $A_{450}$. When the substrate concentration is reduced so that complete hydrolysis of thiophosphoester bonds can be followed spectrophotometrically, there was obtained a biphasic progress curve with a net absorbance change $(A_{324} - A_{450})$ corresponding to a hydrolysis of 112% of the theoretical thiophosphoester bonds. The second phase of the progress curve begins simultaneously with an increase in $A_{450}$.

The progress curve for the hydrolysis of MG(PC) exhibited a lag period before a constant slope of the recorder tracing was reached (Figure 12). The slope of the linear phase, as well as the duration of the lag period was dependent on the substrate concentration. Reduction of the MG(PC) concentration to obtain the entire progress curve gave the lower curve in Figure 12. There appear to be two lag periods. The linear phase shown in the upper curve is terminated after about 40% of the substrate had
Figure 11. Progress curves for the hydrolysis of ME(PC). The plots are recorder tracings of absorbance versus time for incubations of *C. perfringens* phospholipase C with different ME(PC) concentrations in the presence of 0.6 mM DTP and 40 mM CaCl₂. The lower tracing is the complete progress curve for the hydrolysis of 0.05 mM ME(PC). Changes in turbidity were monitored at 450 nm.
Figure 11.
Figure 12. Progress curves for the hydrolysis of MG(PO). Incubation conditions are the same as described in Figure 11.
0.5mM / 0.2mM Mg (PC)

Figure 12.
been hydrolyzed. After 60% hydrolysis, a change in $A_{450}$ signaled a second burst of activity. The net absorbance change corresponded to hydrolysis of 104% of the theoretical content of thiophosphoester bonds. Rates of hydrolysis for this substrate were calculated from the slope of the linear phase following the first lag period.

Linear enzyme dependencies passing through the origin were obtained for both substrates (Figure 13). The slopes of these plots were found to vary with the DTP or DTNB concentration. Since this is a coupled assay system, rate control by the thiol reagent would be expected if reaction with free thiols became rate limiting. This would appear kinetically as an enzyme dependence which was zero order with respect to enzyme at low DTP concentrations. For the family of curves in Figure 13, however, a first order dependence on enzyme was observed at each concentration of DTP.

The stimulatory effect of DTP was verified by comparing rates from spectrophotometric assays with rates from water soluble phosphorus assays. As shown in Table 1, the rates measured by the two types of assays for both substrates are similar. There is a five fold stimulation of the rate of hydrolysis of both ME(PC) and MG(PC) by 0.5 mM DTP as compared to the same incubation in the absence of DTP. In comparison, the hydrolysis of didecanoyl lecithin was stimulated by 50% with 0.5 mM DTP.
Figure 13. Enzyme dependency curves for the reaction of C. perfringens phospholipase C with ME(PC) and MG(PC) at different DTP concentrations. Substrate concentration: 0.5 mM; CaCl$_2$: 25 mM with ME(PC), 40 mM with MG(PC).
Table 1. Hydrolysis rates of ME(PC) and MG(PC) by C. perfringens phospholipase C by spectrophotometric and water soluble phosphorus assay methods at different DTP concentrations. Rates of hydrolysis of didecanoyl lecithin (DDL) under the same conditions are also compared. Rates are expressed as nmol/h/10 ug powder.

<table>
<thead>
<tr>
<th>DTP(mM)</th>
<th>ME(PC)</th>
<th>MG(PC)</th>
<th>DDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>spec.</td>
<td>P</td>
</tr>
<tr>
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<td>47</td>
<td>141</td>
</tr>
<tr>
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</tr>
<tr>
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<td>97</td>
<td>423</td>
</tr>
<tr>
<td>0.50</td>
<td>211</td>
<td>223</td>
<td>638</td>
</tr>
</tbody>
</table>
and by only 20% with 0.5 mM DTNB. DTNB was also less efficient at stimulating the hydrolysis of thiophosphoester substrates. 0.5 mM DTNB increased the rate of MG(PC) hydrolysis by 350% compared to 500% for DTP. A lower enzyme concentration was used to measure the hydrolysis of didecanoyl lecithin than for MG(PC) so that the initial rates measured by phosphorus assay were similar.

Reciprocal plots of varying substrate at fixed DTP concentrations intersected on the abscissa (Figure 14). Likewise, reciprocal plots of varying DTP concentrations at fixed substrate intersected near the abscissa (Figure 15). Replots were made of intercepts and slopes of the reciprocal plots versus the reciprocal of the non-varied component as described by Boyer (1970). Linear replots were obtained from the family of curves in Figure 15, as expected for a sequential bireactant mechanism, from which $K_{ME(PC)}$, $K_{DTP}$, $K_{iDTP}$, and $V_{max}$ were obtained (Figure 16). Parabolic slope and intercept replots from the curves in Figure 14 indicated that the stimulatory mechanism is more complicated than a simple bireactant scheme, and a detailed kinetic investigation will require purified enzyme. Nevertheless, the kinetic parameters calculated from Figure 15 provide a working relationship between the concentrations of ME(PC) and DTP and their effect on the reaction velocity. Normal assay conditions employ 0.6 mM DTP and 1 mM ME(PC). Both
Figure 14. Reciprocal plots of velocity of C. perfringens phospholipase C hydrolysis of varying ME(PC) concentrations at fixed DTP concentrations. Incubations did not contain CaCl₂.
Figure 15. Reciprocal plots of velocity of C. perfringens phospholipase C hydrolysis of fixed ME(PC) concentrations with varying concentrations of DTP. Incubations did not contain CaCl₂.
$K_{ME(PC)} = 1.11 \text{ mM}$

$K_{DTP} = 0.8 \text{ mM}$ (Michaelis constant)

$K_{iDTP} = 0.45 \text{ mM}$ (dissociation constant)

$V_{max} = 400 \text{ nmoles/h/10 ug}$

Figure 16. Slope and intercept replots of Figure 15 as described by Boyer (1970). Replots of Figure 14 are nonlinear.
are near their Michaelis constants, and experimental error in pipetting both reagents will cause poor precision. The $V_{\text{max}}$ value of 400 nmoles/hour is the predicted velocity when both substrate and DTP are saturating, and is 40% higher than the observed value with 2.5 mM DTP.

Reaction products formed during the hydrolysis of thiophosphoester substrates in the presence of DTP were examined as a possible source of stimulation. Incubations were conducted as described in Figure 17 with varying DTP/ME(PC) ratios. After all of the substrate had been hydrolyzed, as judged by the absorbance change at 324 nm, products were extracted with chloroform-methanol (2:1) and analyzed by thin layer chromatography. Although one of the incubations contained only enough DTP to react with one-half the product thiol, it showed the same change in $A_{324}$ as incubations containing an excess of DTP. TLC $R_f$ values of the neutral lipid fraction of the extracts were compared with authentic samples of the product glyceride thiol and its oxidation product, the symmetrical glyceride disulfide. The incubation without DTP produced major amounts of glyceride thiol and trace amounts of glyceride disulfide. All incubations containing DTP, regardless of the concentration, produced only the disulfide. The size of the spot is not diminished by large excesses of DTP, and it therefore seems unlikely that major amounts of the mixed disulfide from glyceride thiol and thiopyridine
Figure 17. Thin layer chromatogram (silica gel G) of reaction products from incubation of C. perfringens phospholipase C with 0.1 mM ME(PC) and different DTP concentrations. Reactions were allowed to proceed to completion and the products then extracted with chloroform-methanol. Glyceride thiol and disulfide standards were prepared synthetically (Cox et al., submitted). Solvent system: hexane-diethyl ether-acetic acid, 80:20:1 (V/V).
are formed as a final product. On tlc in this acidic solvent system, the mixed disulfide should remain near the origin. The large spot at the origin is probably DTP.

When assayed in the absence of DTP, we observed mild stimulation for the hydrolysis of ME(PC) with an optimum around 4 mM (Figure 18). In the presence of DTP, there was a much more pronounced Ca$^{2+}$ stimulation that had no optimum up to 40 mM Ca$^{2+}$.

Reciprocal plots of varying ME(PC) at fixed CaCl$_2$ concentrations revealed that the Ca$^{2+}$ stimulation was due to a $K_m$ effect, with mild uncompetitive inhibition occurring with 100 mM Ca$^{2+}$ (Figure 19). Kinetic parameters for each set of data were determined by direct linear plots and are presented in Table 2. Reciprocal plots of varying Ca$^{2+}$ at fixed ME(PC) concentrations are shown in Figure 20. The nonlinearity of these plots indicates the possibility of more than one enzyme or of a complex mechanism for stimulation. Slope replots of Figures 19 and 20 pass through the origin whilst the intercept replots are horizontal. It is clear that saturating levels of either substrate or Ca$^{2+}$ abolish the enzyme dependence on the other component.

Increasing Ca$^{2+}$ concentrations were also found to shorten the duration of the lag period for MG(PC) in agreement with the findings of Klein et al. (1975) (Table 3). The duration of the lag period is the elapsed time from enzyme addition to the linear phase of the progress
Figure 18. Ca$^{2+}$ dependency of C. perfringens phospholipase C for the hydrolysis of ME(PC) with different concentrations of DTP. CaCl$_2$ concentration is the concentration of added CaCl$_2$. Relative rates are expressed in terms of the measured rate for a given DTP concentration with no added CaCl$_2$. 
Figure 19. Reciprocal plots of velocity of C. perfringens phospholipase C hydrolysis of varying ME(PC) at fixed CaCl$_2$ concentrations. Incubations contained 0.6 mM DTP.
Table 2. Effect of Ca$^{2+}$ on the $K_m$ and $V_{max}$ of C. perfringens phospholipase C for ME(PC) and MG(PC). Values were obtained from direct linear plots (Eisen-thal and Cornish-Bowden, 1974) of the data in Figure 19 for ME(PC). Incubations contained 0.6 mM DTP.

<table>
<thead>
<tr>
<th>Ca$^{2+}$ (mM)</th>
<th>ME(PC)</th>
<th>MG(PC)</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ (uM)</td>
<td>$V_{max}$ (nmol/h)</td>
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<tr>
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</table>
Figure 20. Reciprocal plots of velocity of \textit{C. perfringens} phospholipase C hydrolysis of fixed ME (PC) concentrations with varying CaCl$_2$ concentrations. Incubations contained 0.6 mM EDTA. 

\textbf{Note:} ME (PC) concentrations were fixed while CaCl$_2$ concentrations varied. The reciprocal plots show the relationship between velocity (nmoles/h/10 ug) and CaCl$_2$ concentration (mM$^{-1}$).
Table 3. Effect of Ca\(^{2+}\) and MG(PC) concentrations on the duration of the lag period. Ca\(^{2+}\) effects were measured in the presence of 0.5 mM MG(PC), and MG(PC) effects were measured in the presence of 2 mM CaCl\(_2\). Lag period duration was determined as in Figure 21. All incubations contained 0.6 mM DTP.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Concentration (mM)</th>
<th>Lag Period (min)</th>
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<tbody>
<tr>
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<td></td>
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<tr>
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<tr>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Ca(^{2+})</td>
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</tr>
<tr>
<td></td>
<td>10.0</td>
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</tr>
<tr>
<td></td>
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<td>14</td>
</tr>
<tr>
<td></td>
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<td>26</td>
</tr>
<tr>
<td></td>
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<td>50</td>
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</tbody>
</table>
curve (Figure 21). Increasing temperature shortens the lag, but DTP had no effect. As with ME(PC), a $K_m$ effect was observed for Ca$^{2+}$ stimulation of MG(PC) hydrolysis as measured from the linear phase of the progress curve (Table 2).

Preincubation of enzyme and Ca$^{2+}$ or of MG(PC) and Ca$^{2+}$ had no effect on the lag period. When the enzyme was treated with EDTA and then dialyzed against distilled water, an inactive form was obtained that required Zn$^{2+}$ for reactivation. Preincubation of the inactive enzyme with both MG(PC) and Ca$^{2+}$ before adding Zn$^{2+}$ to start the reaction also had no effect on the lag (Figure 21).

As expected from the greater affinity of EDTA for Zn$^{2+}$ compared to Ca$^{2+}$, addition of EDTA in sufficient amounts to bind all of the Ca$^{2+}$ from an enzyme Ca$^{2+}$ mixture resulted in complete inhibition. Reactivation required titration of EDTA with an excess of Zn$^{2+}$. On the other hand, if the same concentration of EDTA was added to an E,S,Ca$^{2+}$ mixture, only partial inhibition was observed. The degree of inhibition was approximately the same as predicted from Ca$^{2+}$ dependence curves, and is probably due to a reduction of the Ca$^{2+}$ concentration. The progress curves were still linear (albeit with a smaller slope) and

---

1 The association constant $(K = (\text{EDTA}^{2-})/(\text{EDTA}^{4-}) (\text{M}^{2+}))$ of EDTA for Zn$^{2+}$ is $10^6$ times greater than that for Ca$^{2+}$. 


Figure 21. Effect of preincubation of EDTA treated C. perfringens phospholipase C with MG(PC) and Ca$^{2+}$. Incubations contained 0.5 mM MG(PC), 0.6 mM DTP, 10 mM CaCl$_2$, and 1 mM Zn(OAc)$_2$ in 0.1 M Tris buffer, pH 7.0. The long arrow indicates the addition of enzyme and CaCl$_2$ to MG(PC). Short arrows represent the addition of Zn(OAc)$_2$. The lag period is the elapsed time from Zn$^{2+}$ addition to the linear phase of the progress curve.
Figure 21.
showed no signs of gradual enzyme inactivation. Complete inhibition required EDTA in excess of Ca\textsuperscript{2+}. It therefore appears that the enzyme forms a tight complex with substrate and Ca\textsuperscript{2+} and is protected from EDTA. Both substrates gave the same inhibition pattern, and it mattered not whether the EDTA was added during the lag period or during the linear phase of the hydrolysis of MG(PC).

Besides the first lag period for the hydrolysis of MG(PC) there is another pause occurring around 40\% substrate hydrolysis which has not been previously reported for lecithin substrates (Figure 12). A comparison of the progress curves for the hydrolysis of rac-MG(PC) and D-MG-(PC) reveals that the discontinuity for the racemate is due to preferential hydrolysis of the L-isomer (Figure 22). The reaction then slows down before entering into the final burst phase of activity. This latter phase was observed with both ME(PC) and MG(PC) and is associated with a change in turbidity. It occurs at 60-70\% hydrolysis of MG(PC) and 45-50\% hydrolysis of ME(PC). The percentage hydrolysis of substrate at which the burst occurs is invariant with the substrate concentration. This eliminates the possibility that the burst is related to the cmc. The percentage hydrolysis was also not altered by the incorporation of cationic detergents (hexadecyltrimethylammonium bromide or cetylpyridinium bromide) into the micelles of ME(PC), the
Figure 22. Comparison of progress curves for the hydrolysis of D,L-MG(PC), D-MG(PC), and ME(PC) by *C. perfringens* phospholipase C.
Figure 22.
Ca\textsuperscript{2+} concentration, or the substitution of DTNB for DTP, and therefore is not a function of surface charge.

Regarding the substrate specificity of the enzyme, we found that it is mildly stereoselective for the naturally occurring L-isomer of MG(PC). The $K_m$ and $V_{\text{max}}$ for MG(PC) and ME(PC) can be compared in Table 2. Whereas the $K_m$ for ME(PC) is 4 times lower than that for MG(PC), the $V_{\text{max}}$ for MG(PC) is about 4.5 times greater. Substrate dependency plots do not reveal any discontinuities in the region of the cmc (ME(PC) cmc=15 uM, MG(PC) cmc=25 uM) (Figure 23). The enzyme slowly hydrolyzes MG(PE). Rates were determined in the absence of added Ca\textsuperscript{2+} because of its destabilizing effect on the MG(PE) substrate. Under identical conditions, MG(PC) was hydrolyzed 35 times faster than MG(PE).

\textit{Bacillus cereus} phospholipase C. As with \textit{C. perfringens} phospholipase C, treatment of the three thiophosphoester substrates with \textit{B. cereus} phospholipase C in the presence of DTP resulted in an increase in absorbance at 324 nm (Figure 24). None of the progress curves exhibited a lag period. With MG(PC), the hydrolysis proceeded rapidly until 50% of the substrate had been hydrolyzed and then assumed a rate equal to that for the hydrolysis of the D-isomer alone. The initial rate of hydrolysis of the racemate was 30 times greater than that for the D-isomer. Hydrolysis of MG(PE) proceeded to give an absorbance change corresponding to a release of 95% of the theoretical
Figure 23. Substrate dependency plots for the hydrolysis of MG(PC) and ME(PC) by *C. perfringens* phospholipase C in the presence of 25 mM CaCl₂. ME(PC) incubations contained one-half the enzyme that MG(PC) incubations contained. $K_m$ values were determined by direct linear plots (Eisenthal and Cornish-Bowden, 1974).
Figure 23.

Velocity (nmoles/hr)

- ME (PC) $K_m \sim 55\mu M$
- MG (PC) $K_m \sim 220\mu M$
Figure 24. Progress curves for the hydrolysis of MG(PE), D,L-MG(PC), and D-MG(PC) by B. cereus phospholipase C. Incubations contained 0.05 mM substrate and 0.6 mM DTP.
Figure 24.

95.4% of theoretical -SH

D, L-MG(PC)

D-MG(PC)

75.6%

50.5%

time (min)

absorbance
thiophosphoester. The progress curve seemed biphasic, and extrapolation of the linear phases gave an intersection at 76% hydrolysis. This is very close to the value of 74% for the content of L-isomer in the MG(PE) determined by stereochiometric analysis with phospholipase A2 (Cox et al., submitted). The initial rate was approximately 20 times faster than the slower phase. A progress curve for the hydrolysis of ME(PC) (not shown) was similar to the curve obtained for this substrate with C. perfringens phospholipase C, except that the inflection point near 50% hydrolysis was much broader.

Linear enzyme dependencies were obtained with each substrate (Figure 25). It was necessary to use very low concentrations of protein for MG(PE) to obtain a first order relationship, and this was probably due to the poor quality of the substrate suspension. As mentioned earlier, B. cereus phospholipase C was also stimulated by increasing concentrations of either DTP or DTNB, but approximately twice the concentration of DTP was required to achieve the same degree of stimulation as with C. perfringens phospholipase C.

The enzyme was not stimulated by Ca$^{2+}$, but it was inhibited by EDTA. When EDTA was added to the enzyme substrate mixture, the rate slowly declined until the enzyme was inactivated.
Figure 25. Enzyme dependency curves for the hydrolysis of ME(PC), MG(PC), and MG(PE) by B. cereus phospholipase C. Incubations contained the indicated substrate concentration and 0.6 mM DTP.
Figure 26. Substrate dependency curves for the hydrolysis of MG(PC), MG(PE), and ME(PC) by B. cereus phospholipase C. Incubations contained the indicated amount of enzyme and 0.6 mM DTP.
Substrate dependency plots were obtained for each substrate (Figure 26). Discontinuities in the curves at low substrate concentrations occurred in the same vicinity as the cmc. Even if the data was corrected for hydrolysis of water soluble substrate (Bonsen et al., 1972), the curves did not fit Michaelis-Menten kinetics. Visual inspection of the curves in Figure 26 indicates that hydrolysis of substrates below the cmc is a significant portion of the observed rate. This is especially true with MG(PE), where the rate of hydrolysis at saturating levels of substrate is only about twice the rate at the cmc (10 uM). Relative rates for the hydrolysis of ME(PC), MG(PE), and MG(PC) at 0.2 mM substrate concentration are 18, 3.8, and 1 respectively. Under saturating conditions the rates for MG(PC) and MG(PE) are similar.
Discussion

Both *C. perfringens* and *B. cereus* phospholipase C hydrolyze the thiophosphoester linkage of these synthetic phospholipids. The relative rate of hydrolysis of didecanoyl lecithin compared to the thiophosphoester analog is 17 for *C. perfringens* and 2 for *B. cereus* phospholipase C as determined by analysis of water soluble phosphate. From kinetic data on the acid and base hydrolysis of thiophosphoesters, one would expect this group to be hydrolyzed more rapidly than oxyphosphoester (Bruice and Benkovic, 1966). The slowness of the reaction and the difference between the enzymes suggests that either the size of the sulfur atom or its effect on the charge distribution can interfere with the enzyme catalysis, and that the extent of the interference will vary from enzyme to enzyme. TLC analysis of reaction products in the absence of DTP showed a reduction of phospholipid and an increase of glyceride thiol. Hydrolysis therefore proceeds by an S-P rather than C-S cleavage mechanism.

The course of the reaction could be followed spectrophotometrically with either DTP or DTNB. *C. perfringens* phospholipase C hydrolyzes both MG(PC) and ME(PC) to release approximately theoretical amounts of sulfhydryl groups. Neither phospholipase A₁ (*Rhizopus delemar* lipase; Cox, 1977) nor phospholipase A₂ reacted with either substrate to liberate thiol. Turbidity changes
were small for total hydrolysis of low substrate concentrations, and nonexistent during the early stages of hydrolysis for all substrate concentrations used during these assays. These results were taken as proof that substrate hydrolysis occurs and that the glyceride thiol is reactive with water soluble reagents. They also corroborate the isomeric purity of the chemically synthesized substrates, and hence, the specificity for phospholipase C.

During the course of establishing linear enzyme dependencies with MG(PC) and ME(PC) for both enzymes, we observed that the slopes of these plots were affected by the concentration of the thiol reagent (Figure 13). The greatest stimulation was observed with DTP and C. perfringens phospholipase C, and this system was selected for closer analysis. The stimulation was proved to be real by comparison of water soluble phosphate and spectrophotometric assays (Table 1). The much greater stimulation of MG(PC) hydrolysis compared to the oxyphosphoester analog, didecanoyl lecithin, indicates a specific activation mechanism dependent on either the thiophosphoester linkage itself or on the presence of the glyceride thiol product.

Several reports indicate that C. perfringens phospholipase C requires a positive substrate surface charge (Bangham and Dawson, 1962; Dawson, 1976; Klein et al., 1975). Either Ca\textsuperscript{2+} ions or cationic surfactants can be
used for this purpose. Although thiophosphoric acids are slightly more acidic than oxyphosphoric acids (the pKa of S-n-butylphosphorothioate is 1.0 at 25°C compared to 1.9 for the oxygen analog (Bruice and Benkovic, 1966)), the charge difference between the polar head groups of MG(PC) and didecanoyl lecithin with the bulk solution at pH 7 is predicted to be very small. Since the stimulatory effect of DTP for both lecithin and thiophosphoester substrate hydrolysis cannot be matched by equivalent concentrations of DTNB, it is possible that the extra stimulating effect of DTP is derived from its ability to increase the positive surface charge of the substrate.

However, tlc analysis of reaction products formed by ME(PC) hydrolysis in the presence of DTP suggests that DTP partitioning into the micellar phase is minimal (Figure 17). Even in the presence of large excesses of DTP, the major end product of substrate hydrolysis is the symmetrical disulfide formed by disulfide exchange reactions (Figure 27). The faster reaction of glyceride thiol with the thiopyridine-glyceride thiol disulfide probably results from a proximity effect. The mixed disulfide remains bound in the micellar phase and represents a greater effective concentration of reactive disulfide than DTP in the bulk solution. In the vicinity of the enzyme, rapid utilization of micelle bound DTP would probably result in the formation of a DTP lateral
Figure 27. Proposed mechanism for the formation of symmetrical glyceride disulfides during the hydrolysis of ME(PC) by *C. perfringens* phospholipase C.
concentration gradient; these results therefore do not eliminate the possibility that DTP associates with the micelle surface.

Reciprocal plots of varying ME(PC) or MG(PC) at fixed DTP concentrations in the absence of added Ca$^{2+}$ demonstrate that DTP acts to increase the $V_{\text{max}}$ by a saturable mechanism (Figures 14, 15, 16). Because both DTP and DTNB are able to stimulate but have opposite charge, it is unlikely that the major mechanism for rate stimulation is an alteration of surface charge or a reversible noncovalent binding by the enzyme. It is possible that an enzyme sulfhydryl group is oxidized by DTP, and that this might stimulate the hydrolysis of MG(PC) relative to didecanoyl lecithin. However, such an effect should be time dependent, and preincubation of the enzyme and DTP did not increase the hydrolysis rate of ME(PC).

An alternative explanation is that the activation mechanism involves the glyceride thiol product. The enzyme must associate with the surface of the micelle to have access to the vast majority of substrate molecules. Particularly in the vicinity of the micelle bound enzyme, there will be a high concentration of thiols, mixed disulfides, and symmetrical disulfides. An enzyme disulfide bond may be oriented to allow facile disulfide exchange reactions to produce reduced enzyme, enzyme-enzyme disulfide, enzyme-thiopyridine disulfide, and
enzyme-glyceride thiol disulfide. If one of these species is more active than the unmodified enzyme, it would result in DTP or DTNB stimulation specific for thiophosphoester substrates. Increasing concentrations of thiol reagent might shift the distribution of modified enzyme and thereby account for the saturation effect. \textit{C. perfringens} phospholipase C is reported to have six cysteine residues that probably exist as disulfides in the native enzyme (Shemanova et al., 1968, as quoted by Brockerhoff and Jensen, 1974).

\textit{B. cereus} phospholipase C is also stimulated by DTP, but to a lesser extent than \textit{C. perfringens} phospholipase C. Otnaess et al. (1977) has determined that \textit{B. cereus} enzyme contains a single cysteine residue that is not reactive with DTNB even after harsh treatments of heat and denaturants or following treatment with dithioerythritol. However, Kleiman and Lands (1969) found the partially purified enzyme to be inhibited by 1 mM dithiothreitol.

DTP also modified the shape of Ca\textsuperscript{2+} dependency curves of \textit{C. perfringens} phospholipase C with ME(PC). In the absence of DTP, the Ca\textsuperscript{2+} dependency curves had a maximum at 4 mM CaCl\textsubscript{2}, in agreement with the results of other investigators using purified enzymes (Bangham and Dawson, 1962; Yamakawa and Ohsaka, 1977). The high rate in the absence of Ca\textsuperscript{2+} could result from either endogenous Ca\textsuperscript{2+} in the enzyme preparation, the fact that this substrate
exhibits no Ca$^{2+}$ dependent lag period, or the activity of a nonspecific sphingomyelinase inhibitable by 1 mM CaCl$_2$. In the presence of DTP, Ca$^{2+}$ was strongly stimulatory and exhibited a saturation effect with no optimal concentration. The ability of Ca$^{2+}$ to stimulate was dependent on the DTP concentration, and the two acted synergistically. For a fixed concentration of DTP, Ca$^{2+}$ stimulation was manifested as a strict K$_m$ effect.

In contrast, Ca$^{2+}$ stimulated MG(PC) hydrolysis by two separate mechanisms. It not only reduced the substrate K$_m$, as determined from rate measurements made from the linear portion of the progress curve, but it also shortened the lag period. The inability of DTP to affect the lag and the fact that ME(PC) exhibits a K$_m$ effect with no lag period serve to separate the two stimulatory mechanisms. On the other hand, both Ca$^{2+}$ effects appear to be eliminated by saturating levels of substrate. Our findings that increasing concentrations of both substrate and Ca$^{2+}$ decreased the lag period agree with those of Klein et al. (1975). We also found that the lag was shortened by heat and unaffected by DTP. The inability of DTP to affect the lag compared to its cooperative action with Ca$^{2+}$ on the substrate K$_m$, as well as the fact that ME(PC) exhibits a Ca$^{2+}$ dependent K$_m$ effect but has no lag period, serves to separate the two Ca$^{2+}$ stimulatory mechanisms for MG(PC) hydrolysis by C. perfringens phospholipase C.
Preincubation experiments indicated that the lag does not result from E-S or S-Ca\(^{2+}\) complex formation. Preincubation of the Zn\(^{2+}\) free enzyme (*C. perfringens* phospholipase C probably requires Zn\(^{2+}\) as an obligatory cofactor (Kurioka and Matsuda, 1976; Ispolatovskaya, 1970, 1971 as reviewed by Brockerhoff and Jensen, 1974)) with MG(PC) and Ca\(^{2+}\) had no effect on the lag period after the reaction was started by adding Zn\(^{2+}\). These experiments do not eliminate the possibility that the lag period results from the slow formation of an E-S-Ca\(^{2+}\) complex, but they indicate that the Zn\(^{2+}\) containing enzyme must be present in order for the causative event to occur.

Results from EDTA inhibition experiments of *C. perfringens* phospholipase C support the existence of an E-S-Ca\(^{2+}\) complex. Thus, the addition of small amounts of EDTA insufficient to bind all the Ca\(^{2+}\) from an enzyme, Ca\(^{2+}\) mixture resulted in rapid enzyme inactivation. However, if the substrate was added prior to EDTA, the enzyme was protected against complete inactivation and only partially inhibited. Total inhibition required EDTA in excess of Ca\(^{2+}\). Partial inhibition probably is due to reduction of the Ca\(^{2+}\) concentration, which has the effect of increasing the substrate \(K_m\). Although only MG(PC) exhibits a lag period, both substrates exhibited the same EDTA inhibition patterns. With MG(PC), the pattern was the same regardless of whether EDTA was added during or
after the lag period. Enzyme protection against EDTA could result from active site occupation which only occurs in the presence of both Ca\(^{2+}\) and substrate, and does not necessarily imply the formation of a ternary complex distinct from active site turnover.

No lag period was observed for ME(PC) hydrolysis even in the absence of Ca\(^{2+}\) or with low substrate concentrations. This substrate forms smaller particles and has a greater average surface area than MG(PC) as indicated by turbidity measurements. The absence of a lag period may be reflective of a greater effective substrate concentration with ME(PC) compared to MG(PC) for the same substrate molar concentration, or it could result from the different molecular or plurimolecular structure of ME(PC) compared to MG(PC). Jain and Rafael (1978) observed that the lag period for porcine pancreatic phospholipase A\(_2\) hydrolysis of lecithin is dependent on the substrate plurimolecular structure. Multilamellar vesicles exhibited a lag whilst unilamellar vesicles did not.

The linear phase of the MG(PC) progress curve with \textit{C. perfringens} phospholipase C is terminated after about 40\% hydrolysis. We attributed the ensuing rate decline to depletion of the preferred stereoisomer. The final increase in activity occurs after 60-70\% hydrolysis with MG(PC) and 45-50\% hydrolysis with ME(PC), and it seems to be related to a change of the physical state of the
substrate to a more active form. Bangham and Dawson (1962) observed a temporary surface charge reversal at 60-70% hydrolysis of lecithin substrate which resulted in inhibition of \textit{C. perfringens} phospholipase C. In our assay, the products are symmetrical glyceride disulfides rather than diglycerides, and may incorporate more efficiently into the hydrophobic core of the micelles. The structure of partially hydrolyzed substrate micelles should begin to resemble that of a chylomicron or very low density lipoprotein. The surface tension will become lower as substrate molecules are lost into the core, and a combination of these structural changes may cause activation during the course of the hydrolysis. \textit{C. perfringens} phospholipase C hydrolyzes low density lipoproteins, and reduced surface tension has been reported to activate the enzyme (Bangham and Dawson, 1962).

Previous reports on the substrate specificity of \textit{C. perfringens} phospholipase C indicate that the enzyme is either inactive or only slightly active with MG(PE) (Bangham and Dawson, 1962; van Deenen et al., 1961; Pastan et al., 1968; Stahl, 1973; Dyatlovitskaya, 1967). With our assay, the hydrolysis of MG(PC) was 35 times more rapid than the hydrolysis of MG(PE). The purified enzyme is reported to hydrolyze dipalmitoyl lecithin about seven times faster than 1-acylGPC in a diethyl ether system (Stahl, 1973). The $V_{\text{max}}$ for MG(PC) in an aqueous
emulsion is 4.5 times greater than that for ME(PCA).

Precautions must be taken when interpreting substrate specificity from the hydrolysis of MG(PCA), MG(PE), and ME(PCA). Both C. perfringens and B. cereus phospholipase C have some degree of stereoselectivity. Whilst ME(PCA) has no asymmetric center, MG(PE) is enriched in the naturally occurring L-isomer, and MG(PCA) is a racemate. There are also differences in the physical state of each substrate. MG(PCA) forms mixtures of micelles and lamellar structures upon sonication, whereas ME(PCA) is entirely micellar. MG(PE) dispersed poorly, and was actually a microcrystalline dispersion. Both MG substrates contain decanoic acid, but ME(PCA) contains hexadecanoic acid. This substrate is also not directly comparable to the lysolecithin used in so many substrate specificity studies because it is an analog of 2-acylGPC. The lysolecithin commonly used is prepared by the action of phospholipase A₂ on lecithin, and is therefore the 1-acyl isomer.

This difference is especially apparent with B. cereus phospholipase C hydrolysis of ME(PCA). This enzyme is reported to have maximal rates with phosphatidyl choline and to hydrolyze phosphatidyl ethanolamine only slightly more slowly (Otnaess et al., 1977; Roberts et al., 1978; Zwaal et al., 1971). It does not hydrolyze 1-acylGPC
(Kleiman and Lands, 1969; Otnaess et al., 1977), but it does hydrolyze 2-acylGPC (de Haas and van Deenen, 1965). ME(PC) is structurally more similar to 2-acylGPC than 1-acylGPC, and it is hydrolyzed more rapidly than either MG(PE) or MG(PC). *B. cereus* phospholipase C is more stereoselective than the *C. perfringens* enzyme, hydrolyzing the racemic MG(PC) 30 times faster than the D-isomer alone. Roeleffson et al. (1971) found that the enzyme hydrolyzed 1,2-diheptanoylGPC 16 times faster than the 2,3-isomer. Roberts et al. (1978) have recently examined the substrate specificity of *B. cereus* in a Triton X-100 system to eliminate the difference in substrate superstructure between phosphatidyl choline and phosphatidyl ethanolamine. In their system, PE was hydrolyzed at about two-thirds the rate of PC. Under saturating conditions, MG(PE) and MG(PC) were estimated to be hydrolyzed at similar rates. Both enzymes hydrolyzed the substrates at concentrations below their cmc, and the *B. cereus* enzyme was particularly active. For example, the corrected rate for the hydrolysis of MG(PE) under saturating conditions is only about equal to the value at the cmc. This is in agreement with the results of Otnaess et al. (1977), who found that dicaproyl lecithin at concentrations below the cmc had a 5-20 fold higher $V_{\text{max}}$ than dipalmitoyl lecithin.

Thus, the spectrophotometric assay has provided results which have confirmed and elaborated on the results of other
investigators using alternative assay methods. The measured rates are identical with phosphorus assay rates, and the progress curves portray a realistic view of rate as a function of time. We have tested some of the potential applications with crude preparations of bacterial phospholipase C and found that it is a useful tool for kinetic and mechanistic studies.
CHAPTER IV
CHARACTERIZATION OF THE PHOSPHOLIPASE ACTIVITY
OF RHIZOPUS DELEMAR LIPASE AND ITS USE TO
PURIFY CHOLINE AND ETHANOLAMINE PLASMALOGENS

Introduction

Plasmalogens (1-alk-1'-enyl-2-sn-glycerophospholipids) are widely distributed in nature (Horrocks, 1972) and are almost always found as mixtures with diacyl and alkylacyl analogs. Their resistance to phospholipases (Woelk and Porcellati, 1978) and relative abundance of acyl groups that are prostaglandin precursors (Horrocks and Fu, 1978) has led to speculation on their functional role in membranes. Plasmalogen degradation by plasmalogenase is postulated as an important early step in white matter demyelination (Horrocks et al, 1978). A rapid, reproducible method for preparing purified plasmalogens would facilitate research in this area.

Several possibilities exist for the removal of the diacyl glycerophospholipids from mixtures containing plasmalogens. Plasmalogens react more slowly than diacyl glycerophospholipids with methoxide ion (Renkonen, 1963; Ansell and Spanner, 1963; Frosolono and Marsh, 1973),
phospholipase A₂ (Gottfried and Rapport, 1962; Woelk and Debuch, 1971), phospholipase C (Warner and Lands, 1963), and phospholipase D (Lands and Hart, 1965). As discussed by Woelk et al. (1973) each of these purification methods has disadvantages of either low yield, low efficiency, or nongenerality for both choline and ethanolamine plasmalogens. They developed a purification procedure employing pancreatic lipase. After a single incubation of lipase with either bovine heart choline glycerophospholipids or bovine brain ethanolamine glycerophospholipids, they obtained a plasmalogen preparation of 80% purity. Following Florisil column chromatography to remove lyso products, reincubation resulted in the complete hydrolysis of the remaining diacyl phospholipid. Plasmalogens were recovered in an overall yield of 74%. The major disadvantage of this method is that pancreatic lipase must be purified to remove the phospholipase A₂ that is present in commercially available lipase preparations. Furthermore, the double incubation-double column chromatography procedure is time consuming for routine work. Nevertheless, this procedure produces highly purified plasmalogens in good yield and is applicable to both choline and ethanolamine plasmalogens.

*Rhizopus delemar* lipase secretes three lipases into its culture medium with hydrolytic activities toward triglycerides with the same specificity as pancreatic lipase (Iwai and Tsujisaka, 1974; Tsujisaka et al., 1972). The
lipase preparation from Rhizopus arrhizus has phospholipase A1 activity (Slotboom et al., 1970a) and has also been used for the purification of plasmalogens (Paltauf, 1977). We have found that the commercially available lipase from Rhizopus delemar (glyceryl ester hydrolase, EC 3.1.1.3) provides a rapid, reproducible, and convenient route for the preparation of ethanolamine and choline plasmalogens. A preliminary report has appeared (Cox, 1977).
Materials and Methods

Analytical. Lipid phosphorus was determined by the method of Gottfried (1967). Acid-labile and acid-stable fractions of ethanolamine and choline glycerophospholipids were assayed as lipid phosphorus following two-dimensional reactionary TLC (Horrocks, 1968). Acid-stable ethanolamine glycerophospholipids were analyzed for diacylGPE and alkylacylGPE by mild alkaline methanolysis (Horrocks and Sun, 1972). Enol ether contents were assayed by I$_2$ addition (Gottfried and Rapport, 1962) using dihydropyran (Aldrich, Gold Label, Milwaukee, Wisconsin) as standard. Esters were measured as hydroxamate derivatives (Stern and Shapiro, 1953).

TLC. Thin layer chromatographic plates (20 x 20 cm) were prepared from a slurry of 35 gm silica gel G (Em Laboratories, Elmsford, New York) in 67 ml water spread to a thickness of 0.5 mm. Plates were activated a minimum of 30 minutes prior to use. Solvent systems for both first and second dimensions were chloroform/methanol/water (65/25/4). Spots were visualized with I$_2$ vapor.

Substrates. Ethanolamine glycerophospholipids were isolated from both bovine brain white matter and bovine heart (D'Amato et al., 1975). Total lipids were extracted according to Polch et al. (1957), and fractionated on neutral alumina (7 umoles/g) as described by Ansell and Spanner (1963). The ethanolamine glycerophospholipid
fractions from white matter and heart contained 68% and 42% plasmalogens, respectively. Similarly, choline glycerophospholipids were isolated from bovine heart and contained 46% plasmalogen.

Enzyme assay. Rhizopus delemar lipase (glyceryl ester hydrolase, E.C. 3.1.1.3, 600 units/mg, C grade) was purchased from Miles Laboratories (Elkhart, Indiana). Several lot numbers have been used with identical results. Incubation conditions for individual experiments are given in the figures and tables. With the exception of the time course experiment, incubation volumes were 0.2 ml. For detergent and pH studies, emulsions were prepared individually by coevaporation of phospholipid and detergent in small culture tubes under N₂, addition of buffer and CaCl₂ solution, and sonication for 20 seconds at low intensity with tubes immersed in an ice bath using a Bronwill sonicator (Will Scientific, Rochester, New York). Fatty acid-free bovine serum albumin (Pentax, Miles Laboratories, Elkhart, Indiana) was then added and the reaction started by the addition of enzyme. For calcium ion and bovine serum albumin studies, a stock emulsion containing 20 mg ethanolamine glycerophospholipids per ml, 0.2% Triton X-100 and 0.2M sodium 3-(N-morpholino)-propanesulfonate/HCl buffer (MOPS) was prepared by sonication. Aliquots of 0.1 ml were adjusted to a final volume of 0.2 ml. Controls contained no enzyme. For the
time course experiment an incubation mixture of 3.0 ml was sampled at intervals. Reactions were terminated by mixing with four volumes of chloroform-methanol 2/1 (v/v). Portions of the lower phase were taken directly for TLC analysis. Plates were exposed to HCl fumes for two minutes prior to development. Rates were calculated from the decrease in acid-stable phospholipid.

**Positional specificity.** A mixture of 12 mg diacylGPC and two mg 1-(³H)alkyl-2-acyl-GPC (provided by Dr. G. Goracci) was incubated in a total volume of 3.0 ml containing 0.1 M sodium acetate buffer (pH 5.6), 5 mM CaCl₂, 2 mM sodium deoxycholate and 3 mg enzyme. The mixture was shaken for 17 hours at 37° C, and the product was isolated by the method of Bligh and Dyer (1959). A portion was taken for analysis by one-dimensional TLC. The diacylGPC and monoacylGPC bands were scraped into separate sintered glass funnels and eluted with chloroform-methanol-water 45/45/10 (by volume). Samples were taken for phosphorus determination and liquid scintillation counting.

**Preparative incubations.** Incubation volumes have ranged from six to 72 mls containing the following: 10 mg ethanolamine or choline glycerophospholipids per ml, 0.1% Triton X-100 (x/v), 0.5% bovine serum albumin (w/v), 5 mM CaCl₂, 0.1 M MOPS buffer (pH 7.0), and two mg enzyme per ml. The phospholipid and Triton X-100 were coevaporated in the reaction flask under a stream of N₂ or by rotary evaporation.
Buffer and CaCl$_2$ solutions were warmed to 45°C, then added to the lipid residue. The mixture was stirred magnetically until a milky emulsion was obtained. A uniformly fine dispersion is not necessary at this stage, since the lyso reaction product produces a better emulsion as the reaction progresses. Bovine serum albumin and enzyme were then added and the mixture was stirred overnight at room temperature. The lipid was then extracted with 4 volumes of chloroform:methanol 2/1 (v/v), the solvent was evaporated, and the lipid was transferred to a Unisil silicic acid (Clarkson Chemical Co., Williamsport, Pennsylvania) column (15 umoles phosphorus per g silicic acid). With ethanolamine glycerophospholipids, the column was eluted with chloroform, chloroform-methanol 7/1 (v/v), chloroform-methanol 4/1 (v/v), and methanol. Plasmalogens were eluted in the second and third fractions. For choline glycerophospholipids, the solvents were chloroform, chloroform-methanol 2/3 (v/v) and methanol, and plasmalogens eluted in the second fraction.
Results

DiacylGPE is hydrolyzed efficiently by *R. delemar* lipase, but neither ethanolamine plasmalogens nor alkylacyl-GPE showed any significant change in concentration (Figure 28). The lack of a stoichiometric recovery of monoacylGPE with the concurrent increase of water-soluble phosphorus suggests that some lysophospholipase activity is present. The positional specificity of the lipase was verified by incubating a mixture of diacylGPC and $(^3\text{H})$alkylacylGPC. If any phospholipase $A_2$ activity was present, then the monoacylGPC region of the TLC plate should have contained $^3\text{H}$ label. Although the enzyme was active, as indicated by an 80% hydrolysis of the diacylGPC, no $^3\text{H}$ was found in the lyso product.

Detergents have little effect on either the initial rate or the extent of hydrolysis of acid-stable ethanolamine glycerophospholipids after relatively long incubations, even with a 600-fold range of Triton X-100 concentrations (Table 4). Unlike some other phospholipases, the enzyme is not active in the presence of ether.

The lipase activity exhibits a broad pH optimum of 5.5-7.0 for the hydrolysis of diacylGPE with a sharp decrease in activity above pH 7.5 (Figure 29). Since similar values for hydrolysis were obtained with different buffers at the same pH, the lipase activity does not depend on the nature of the buffer. Both calcium ion and
Figure 28. Time course of the hydrolysis of ethanolamine glycerophospholipids by R. delemar lipase. Incubation mixture: 50 mM sodium acetate (pH 5.6), 100 mM NaCl, 5mM CaCl₂, 2 mM sodium deoxycholate, 0.5% bovine serum albumin, 5 mg ethanolamine glycerophospholipids per ml, and 2 mg lipase per ml at 37°C with magnetic stirring.
Table 4. Effects of detergents on the initial rate and extent of hydrolysis of acid stable ethanolamine glycerophospholipids.

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Relative Initial Rate</th>
<th>Relative Extent of Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% Triton X-100</td>
<td>0.9</td>
<td>0.93</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>1.0</td>
<td>1.00</td>
</tr>
<tr>
<td>2.0% Triton X-100</td>
<td>---</td>
<td>0.97</td>
</tr>
<tr>
<td>6.0% Triton X-100</td>
<td>0.6</td>
<td>1.05</td>
</tr>
<tr>
<td>0.1% Tween 20</td>
<td>0.7</td>
<td>1.01</td>
</tr>
<tr>
<td>2 mM NaDOC</td>
<td>1.0</td>
<td>0.99</td>
</tr>
<tr>
<td>7mM NaDoc</td>
<td>1.7</td>
<td>0.99</td>
</tr>
<tr>
<td>Ether/Water</td>
<td>---</td>
<td>0.16</td>
</tr>
<tr>
<td>None</td>
<td>0.6</td>
<td>1.01</td>
</tr>
<tr>
<td>None, without enzyme</td>
<td>0.0</td>
<td>0.08</td>
</tr>
</tbody>
</table>

a. Percentage concentrations are W/V.

b. Standard deviations estimated from ten sets of triplicate samples giving similar percentage hydrolysis are 0.2 for the relative initial rate and 0.03 for the relative extent of hydrolysis.

c. For 0.1% Triton X-100, the initial rate was 9.1% hydrolysis, and the extent of hydrolysis was 76%.

d. The diethyl ether-water mixture was 1/1 (V/V).
Figure 29. Effect of pH on the hydrolysis of acid stable ethanolamine glycerophospholipids. Incubation mixture: 0.1 M buffer, 5 mM CaCl₂, 0.1% Triton X-100, 0.5% bovine serum albumin, 10 mg ethanolamine glycerophospholipids per ml, 1 mg lipase per ml at 37°C in a shaking water bath. Buffers: pH 3.8-5.5, sodium acetate; pH 5.9-6.5, morpholinoethanesulfonic acid; pH 6.9-7.5, morpholinopropanesulfonic acid; pH 7.8-8.7, Tris. Bars indicate the range of duplicate samples.
bovine serum albumin could effect the extent of hydrolysis by binding hydrolyzed fatty acids. If all of the diacylGPE were hydrolyzed, then either 2 mM CaCl$_2$ or 2% bovine serum albumin (w/v) is sufficient to bind the free fatty acids (assuming two fatty acid anions per calcium ion and ten fatty acid binding sites per bovine serum albumin monomer) (Tanford, 1973). Nearly 40% hydrolysis is attained with either (Table 5). In the absence of bovine serum albumin, 1 mM CaCl$_2$ was stimulatory while 10 and 100 mM were inhibitory. In the absence of calcium ion, increasing concentrations of bovine serum albumin gave higher rates. The stimulatory effect of bovine serum albumin was diminished with increasing CaCl$_2$ concentrations. With the exception of the emulsion containing 2.0% bovine serum albumin, all emulsions prepared with 100 mM CaCl$_2$ were unstable.

Preparative incubations with ethanolamine glycerophospholipids gave products that were 94-95% pure ethanolamine plasmalogen preparations according to P assays (Table 6). The deviation from unity for the acid-labile to total phosphorus and enol ether to phosphorus ratios can be explained in part by the alkylacylGPE component, but the ester to phosphorus ratio indicates that diacylGPE is still present. Analysis of the acid-stable ethanolamine glycerophospholipid fraction by alkaline methanolysis revealed that it was 60% alkylacylGPE and 40% diacylGPE. Thus, the overall analysis was 95% alkenylacylGPE, 3% alkylacylGPE, and
Table 5. Effects of Ca\textsuperscript{2+} and bovine serum albumin on the rate of hydrolysis of acid stable ethanolamine glycerophospholipids.

<table>
<thead>
<tr>
<th>CaCl\textsubscript{2}, mM</th>
<th>% bovine serum albumin (w/v)</th>
<th>relative rate\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>0</td>
<td>0.65</td>
<td>0.75</td>
</tr>
<tr>
<td>1</td>
<td>0.80</td>
<td>0.81</td>
</tr>
<tr>
<td>10</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>100</td>
<td>0.22</td>
<td>0.16</td>
</tr>
</tbody>
</table>

\textsuperscript{a} A relative rate of 1.00 corresponds to 58\% hydrolysis. Values are averages duplicate samples with an average range of 0.04. Incubation conditions are identical to those given in Figure 29.
Table 6. Characterization of the ethanolamine glycerophospholipid before and after lipase treatment.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Starting Material</th>
<th>Product Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>enol ether/P</td>
<td>0.72</td>
<td>0.96 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>acid labile P/total P</td>
<td>0.68</td>
<td>0.95 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ester/P</td>
<td>1.29</td>
<td>1.05 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>acid labile P/total P</td>
<td>----</td>
<td>0.94 ± 0.013&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard deviations were calculated from the standard deviations of the numerator and denominator by propagation of random error. These values are from triplicate assays of a single plasmalogen preparation.

<sup>b</sup> This is an average of 11 values from preparative incubations ranging in volume from 6 to 72 ml.
2% diacylGPE. Choline plasmalogen preparations were 94% pure according to P assays after two-dimensional reaction-ary TLC. The recovery of plasmalogens following incubation and column chromatography was consistently between 80 and 90%.
Discussion

The *Rhizopus delemar* lipase offers the same advantages as pancreatic lipase while it avoids the problems of enzyme purification and double incubation. The commercially available, inexpensive, crude enzyme preparation is free of phospholipase A₂ activity. A single incubation with either choline or ethanolamine glycerophospholipids yields a product with a plasmalogen content of 94%. Although the diacyl component was not completely hydrolyzed, it is reduced to about 2% of the ethanolamine glycerophospholipids. For the purpose of using the plasmalogen preparation as an enzyme substrate, this is tolerable as long as the contamination is reproducible. This was found to be so in 11 preparative incubations ranging in volume from 6 to 72 ml. By eliminating the extra incubation, time is saved and the plasmalogen recovery increased to 80-90%, depending on the care taken with the column chromatography. Recently, Paltauf (1977) has briefly reported similar results from *Rhizopus arrhizus*. In order to test the suitability of *R. delemar* lipase for the purification of plasmalogens, initial studies were concerned with the amount and type of phospholipase activity present in the crude enzyme preparation. Both diacylGPE and diacylGPC were hydrolyzed to lysophospholipids, while neither of the plasmalogens was enzymically degraded. The absence of phospholipase A₂ activity was verified using radiolabeled alkylacylGPC. The
major phospholipase activity is type A₁ with a lesser amount of lysophospholipase activity.

In order to improve the efficiency, we examined effects of pH, calcium ion, and bovine serum albumin. The *R. delemar* lipase reaction is reversible as demonstrated by ester formation from glycerol and fatty acid under anhydrous conditions (Tsujisaka et al., 1972). The reverse reaction requires protonated fatty acid substrate; thus, Brockerhoff et al. (1976) were able to exchange fatty acids at the 1-position of diacylGPE and diacylGPC using *R. delemar* lipase at pH 3.4. The pH optimum of the three *R. delemar* lipases with triglyceride is 5.6 (Iwai and Tsujisaka, 1974). Two are unstable above pH 7.0, the third above pH 8.0 (Iwai and Tsujisaka, 1974). With ethanolamine glycerophospholipids as substrate, the lipase has a broad pH optimum of 5.5-7.0. This allows incubations to be performed at neutral pH, reducing both the concentration of protonated fatty acid and the acid-catalyzed hydrolysis of plasmalogen. At pH 5.6, roughly 5% of the plasmalogen was hydrolyzed per hour, whereas less than 10% was hydrolyzed during overnight incubations at pH 7.

Fatty acids may also be removed from the system by complex formation with either calcium ion or BSA. Calcium ion did not stimulate the initial rate of hydrolysis of triglyceride by *R. delemar* lipase, but it prevented
eventual inhibition, presumably by removing fatty acids as insoluble soaps (Tsujisaka et al., 1972). Concentrations up to 0.1 M were not inhibitory. With ethanolamine glycerophospholipids, calcium ion was not a required cofactor, but a concentration sufficient to bind released fatty acid was stimulatory. Higher concentrations were inhibitory, possibly because of emulsion destabilization. Bovine serum albumin is postulated to activate pancreatic lipase by binding fatty acids and preventing enzyme denaturation at the oil/water interface (Brockerhoff and Jensen, 1974). With crude enzyme preparations, it may also protect against the action of proteases.

Preparative incubation conditions therefore included sufficient calcium ion (5 mM CaCl₂) to bind the fatty acid released from glycerophospholipid mixtures containing about 50% plasmalogen, such as bovine heart choline glycerophospholipid. Bovine serum albumin (0.5% w/v) was included for other possible activating effects, and the incubations were conducted at pH 7. Triton X-100 (0.1%, w/v) was selected as detergent since it is easily separable from the product and facilitates the initial substrate emulsification. Overnight incubations at room temperature were most convenient.

In addition to plasmalogen purification, Rhizopus delemar lipase can be used to purify alkylacyl phospholipids as demonstrated here with alkylacylGPC, or to
stereospecifically exchange fatty acids at the one position of diacylGPE or diacylGPC as described by Brockerhoff et al. (1976). It is a convenient substitute for pancreatic lipase especially when neutral or acidic pH is desired.
CHAPTER V

A CONTINUOUS SPECTROPHOTOMETRIC ASSAY FOR PHOSPHOLIPASE A\textsubscript{1} WITH THIOESTER SUBSTRATES: PREPARATION OF SUBSTRATES AND DEVELOPMENT OF THE ASSAY USING RHIZOPUS DELEMAR LIPASE

Introduction

Compared to phospholipase A\textsubscript{2}, phospholipase A\textsubscript{1} is an ill-defined enzyme. One reason for ambiguity is that many investigators assaying for phospholipase activity used phospholipid randomly labelled with radioactive fatty acid, and referred to any esterase activity as phospholipase A\textsubscript{1}. Because of the implicated control by phospholipase A\textsubscript{2} of prostaglandin production (Horrobin, 1978), the properties and distribution of phospholipase A\textsubscript{2} in mammals became a major area of interest and very little attention was devoted to phospholipase A\textsubscript{1}. Another reason for ambiguity is that even if a phospholipase A\textsubscript{1} activity could be firmly identified, there was always the question of whether it was due to a true phospholipase A\textsubscript{1} or a nonspecific triglyceride lipase (Colbeau et al., 1977; Sundaram et al., 1978; Slotboom et al., 1970; Waite et al., 1975). Finally, although there are several commercial sources of
phospholipase $A_2$ and triglyceride lipase, there is not a readily available source of phospholipase $A_1$ specific for phospholipids. All synthetic (Brockerhoff et al., 1976; Slotboom et al., 1970b), purification (Woelk et al., 1973; Cox, 1977; Paltauf, 1978), or structural work with lipids requiring phospholipase $A_1$ activity has relied on the phospholipase activity of the commercial Rhizopus lipases or purified porcine pancreatic lipase. There is no doubt that the commercial availability of phospholipase $A_2$ stimulated its use for membrane structural analysis and facilitated the intensive kinetic studies on $A_2$ binding and hydrolysis of phospholipid (Slotboom et al., 1978; Roberts et al., 1977).

Enzyme assays for phospholipase $A_1$ are generally radiochemical. Radioactive substrates with labeled fatty acid at the 2-position are incubated with enzyme and the incubation products are fractionated by extraction and thin layer chromatography. Lysophospholipid, fatty acid, and starting material are usually counted to differentiate between phospholipase $A_1$ and phospholipase $A_2$. Short cuts have been devised to avoid tlc separation (Scandella and Kornberg, 1971; Sundaram et al., 1978), but the assays are still discontinuous, time consuming, and expensive. Even with sufficient phospholipase $A_1$ activity, many of these enzymes cannot be assayed by titration of released fatty acids because of their neutral to acidic pH optima.
Aarsman et al. (1976) applied the thioester substitution technique to the assay of phospholipase A\textsubscript{2}. These workers prepared a glycol lecithin analog in which a thioester linkage was substituted for the oxyester. Hydrolysis by phospholipase A\textsubscript{2} or lysophospholipase L\textsubscript{2} (Aarsman and van den Bosch, 1977) produced a water soluble thiol which reacted with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to produce color. We extended this concept to the assay of phospholipase C with thiophosphoester substrates (Cox et al., 1979; Cox et al., submitted). This system was more complex than the phospholipase A\textsubscript{2} assay in that the product thiols were not water soluble, but they were still reactive with DTNB or 4,4'-dithiopyridine (DTP).

For the assay of phospholipase A\textsubscript{1}, rac-1-S-2-0-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (XVII) and rac-1-S-2-0-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (XVIII) were synthesized from mercaptoglycerol. The assay was developed with the lipase from Rhizopus delemar, a commercial lipase preparation which has small amounts of phospholipase A\textsubscript{1} and lesser amounts of lysophospholipase activity (Cox, 1977). In this paper, evidence is presented that the lipase and phospholipase activities reside in separate enzymes. This continuous spectrophotometric assay method is sensitive and specific for phospholipase A\textsubscript{1}, and it can be used under acidic conditions.
Materials and Methods

Materials.  *Rhizopus delemar* lipase (glyceryl ester hydrolase, E.C. 3.1.1.3) in both crude (600 U/mg) and purified (6000 U/mg) forms was a generous gift of Miles Laboratories (Elkhart, Indiana). Mercaptoglycerol was purchased from Evans Chemetics (Darien, Connecticut) and fractionally distilled. Decanoyl chloride, palmitoyl chloride, bromoethanol, ethanolamine, and 4,4'-dithiopyridine (DTP) were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). Trimethylamine (anhydrous) was purchased from Eastman Kodak Company (Rochester, New York). 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Sigma Chemical Company (St. Louis, Missouri). Chromatography solvents were reagent grade or better. Pyridine and triethylamine were distilled from BaO, and chloroform and trichloroethylene were washed with water, dried over Na$_2$SO$_4$, and distilled from CaSO$_4$. Tetrahydrofuran was refluxed over Na chips for two hours prior to distillation. Bromoethylphosphorusoxydichloride was prepared from freshly distilled bromoethanol and phosphorusoxytrichloride. Bromoethanol was added to a 3 mol excess of POCl$_3$ in benzene and the solution refluxed for two hours in an exhaust hood. The solution was rotary evaporated at 55° C and the product vacuum distilled in 80% yield (81° C, 0.4 mm Hg). rac-1,2-Didecanoylglycerol was synthesized as described by Jensen and Pitas (1976)
using boric acid to remove the dihydropyran blocking group. This was then converted into the phosphocholine derivative according to Hirt and Berchtold (1958).

**Analytical Methods.** Melting points were determined with a Thomas Hoover Capillary Melting Point Apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tennessee) after drying the samples in vacuo over P$_2$O$_5$ for 24 hours. Lipid phosphorus was determined by the method of Gottfried. Infrared spectra were either obtained neat or as 10% (W/V) solutions in chloroform against a blank cell containing chloroform with a Beckman Model 4230 Infrared Spectrophotometer. Visible and UV spectrophotometric measurements and enzyme assays were performed with a Beckman Model 25 Recording Spectrophotometer equipped with an automatic sample changer.

**Chromatography.** Analytical thin layer chromatography (tlc) was done with 0.5 mm thick layers of silica gel G (EM Laboratories, Elmsford, New York) prepared from a slurry of 35 g gel and 67 ml water. Spots were visualized with either I$_2$ vapor, phosphate spray, phosphate spray reagent (Vaskovsky and Kostetsky, 1968), Dragendorf stain for choline (Beiss, 1964), ninhydrin spray for primary amine (Waldi, 1965), or DTNB (Ellman, 1958) for sulfhydryl groups (5 mM DTNB in ethanol-water, 2:1, pH 7). Unisil silicic acid was a product of Clarkson Chemical Company.
(Williamsport, Pennsylvania). Neutral silica gel for column chromatography was prepared from silicic acid by titrating an aqueous slurry with NaOH to pH 7. The slurry was then suction filtered and dried overnight at 120°C.

**Substrate Suspensions.** A 1 mM emulsion of the phosphatidyl choline (PC) analog (XVII) was prepared by drying 15 umoles of the lipid under N\textsubscript{2} in a 50 ml beaker and suspending in 15 ml buffer by sonication for four minutes at 35% intensity with a Bronwill Biosonik III Sonifier (Will Scientific, Rochester, New York) equipped with a 3/4 inch probe. The beaker was immersed in ice during the sonication. The absorbance at 450 nm (A\textsubscript{450}) of a 0.5 mM emulsion was 0.2. The phosphatidyl ethanolamine (PE) analog (XVIII) gave unstable crystalline dispersions under these conditions, but when small amounts of chloroform were added prior to sonication, stable emulsions with low turbidity were formed (Figure 32). Routine emulsions were therefore prepared by drying 15 umoles of the PE analog (XVIII) from chloroform solution in a beaker under N\textsubscript{2}, followed by heating in a 120°C oven for one minute to completely evaporate the organic solvent. After cooling, 15 ml buffer followed by 10 ul chloroform was added and the mixture sonicated as before. The monopalmitin (MP) (XIX) and monodecanoin (MD) (XX) analogs were suspended as semi-stable microcrystalline dispersions with the aid of didecanoylGPC. Coevaporation of 6 umoles MP (XIX) or
MD(XX) with 6 umoles didecanoylGPC followed by addition of 15 ml buffer and sonication as above gave a suspension with an $A_{450}$ of 0.6 for MP(XIX) and 0.4 for MD(XX) and no significant baseline drift. Sedimentation of MP(XIX) crystals occurs if the suspension is allowed to stand for several hours, but they can be resuspended by vortexing. All suspensions were allowed to age at least one hour at room temperature prior to use.

**Enzyme Assays.** Routine assay conditions for the PC (XVII) and PE (XVIII) analogs included 0.5 mM substrate suspension, 0.6 mM DTP, and enzyme in 0.1 M buffer. Total volume was 1 ml. Reactions were run at room temperature and were started by adding enzyme solution. Assay conditions for the MG analogs (XIX and XX) included 0.2 mM substrate suspension (containing 0.2 mM didecanoylGPC), 5 mM CaCl$_2$, 0.6 mM DTP, and enzyme in 0.1 M buffer. Rates were calculated from the molar absorption coefficient for 4-thiopyridone at 324 nm of $1.98 \times 10^4 M^{-1}cm^{-1}$ (Grassetti and Murray, 1967). Measurements were normally made against air.

**pH Dependence.** Substrate suspensions were prepared in 0.05 M sodium acetate-0.05 M sodium 3-(N-morpholino) propane sulfonate buffer adjusted to pH 7.5 with HCl. The suspensions were then adjusted down in 0.5 pH increments with HCl to pH 4.0. This method introduces a salt gradient, but no NaCl dependence was observed at pH 7.5.
Chemical Syntheses of Thioester Substrates

Preparation of rac-1,2-S,0-didecanoyl-1-mercapto-2,3-propanediol (XVI). Mercaptoglycerol was triacylated with decanoyl chloride to form the triglyceride (XV), which was then partially hydrolyzed with R. _delemon_ lipase (600 U/mg) to obtain the desired diglyceride (XVI) (Figure 30). Mercaptoglycerol (35 mmoles) was dissolved in 100 ml chloroform and 210 mmoles pyridine. Decanoyl chloride (175 mmoles) was added with rapid stirring. After cooling, the mixture was allowed to stand in the dark for 2 days. It was then extracted with 500 ml hexane and washed 3 times with 100 ml portions of water. Rotary evaporation of the organic phase gave 30 g of brown oil. The crude product (5 g) was purified on a 200 g column of silicic acid by elution with a hexane-diethyl ether solvent system (98:2, 95:5, and 85:15). The triglyceride (XV) is obtained in 95% yield as a light yellow oil that migrates just ahead of trimyristin on tlc (hexane-diethyl ether-acetic acid, 90:10:1, \(R_f=0.3\)). The infrared spectrum of the neat oil exhibited the following strong bands: 2970, 2940, 1460, 1375 (C-H); 1740, 1200-1150, 1110, 1050 (ester); and 1695 cm\(^{-1}\) (thioester).

The triglyceride (XV) was partially hydrolyzed with R. _delemon_ lipase using the assay conditions described by Fukumoto et al. (1964). (XV) (10 mmol) was incubated with 5 mg lipase in a volume of 60 ml containing 0.2 M sodium
acetate buffer, pH 5.6, and 10 mM CaCl₂. The mixture was rapidly stirred and the progress of the reaction followed by tlc (hexane-diethyl ether-acetic acid, 60:40:1). After one hour of incubation, five components of the reaction mixture could be detected, and they were tentatively identified as unreacted starting material with \( R_f = 0.98 \), 2,3-O-didecanoyl-1-mercapto-2,3-propanediol with \( R_f = 0.95 \), decanoic acid with \( R_f = 0.68 \), 1,2-SO-didecanoyl-1-mercapto-2,3-propanediol (XVI) with \( R_f = 0.47 \), and 2-O-decanoyl-1-mercapto-2,3-propanediol with \( R_f = 0.26 \). The concentration of (XVI) reached a maximum after about three hours incubation, and the mixture was extracted with four volumes chloroform-methanol (2:1). After rotary evaporation at 30°C, the residual oil was fractionated on a 200 g column of neutral silica gel by elution with hexane-diethyl ether (90:10, 80:20, and 70:30). The desired diglyceride (XVI) elutes in the last fraction in 26% yield. The infrared spectrum of the neat oil was identical to (XV) except for the presence of a strong hydroxyl band at 3450 cm⁻¹ and weaker ester bands. A trace amount of the 1,3-isomer was formed during column chromatography, but it was judged to be less than 5% of the 1,2-isomer (tlc, hexane-diethyl ether-acetic acid, 60:40:1, \( R_f = 0.47 \) for the 1,2-isomer compared to 0.53 for the 1,3-isomer).

**Preparation of rac-1,2-S,0-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (XVII).** The diglyceride (XVI)
(3.45 nmol) was dissolved in 15 ml chloroform and added dropwise to a well stirred solution of 13.8 mmol bromo-ethylphosphorusoxydichloride and 28 mmol triethylamine at 0-5° C. The mixture was stirred at room temperature for 12 hours and then subjected to hydrolysis, trimethylamination, and purification as described by Aarsman et al. (1976). Following the treatment with ion exchange resin, the crude products were taken up in chloroform and eluted from a 100 g column of silicic acid with chloroform-methanol (4:1, 1:1, 2:3, and 1:3). The phosphocholine derivative (XVII) elutes in the last two fractions in 40% yield. On tlc it migrated identically to didecanoylGPC and stained positively for both choline and phosphate groups. TLC (chloroform-methanol-water, 65:35:4) Rf = 0.25. The infrared spectrum of (XVII) is compared to didecanoylGPC in Figure 31. Assignments are as follows: 3300 (bound water); 2970, 2940, 2860, 1470, and 1380 (C-H); 1740, 1170 (ester); 1695 (thioester); 1260, 1095 (P=O); 1060 ((P)OC); 970, 920 (quaternary trimethylammonium salt); 870 (PO(C)); and 820, 720-790 cm⁻¹ (O-P-O). A single elemental analysis yielded the following results:

C_{28}H_{56}O_{7}NSP requires: C 57.8, H 9.7, N 2.4, S 5.5, P 5.3%
found: C 57.6, H 9.7, N 2.4, S 5.6, P 5.4%

**Preparation of rac-1,2-S,0-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (XVIII).** The diglyceride (XVI) (3.45 mmol) was converted to the phosphoethanolamine derivative as described by Eibl (1978). The workup was
modified, however, since the didecanoyl analog failed to precipitate after the addition of acetic acid. The solution was rotary evaporated and purified by chromatography on a 100 g column of silicic acid with a chloroform-methanol solvent system (chloroform-methanol 9:1, 6:1, 5:1, and 4:1). The desired product (XVII) eluted in the last fraction in 30% yield. On tlc, it migrated behind bovine brain ethanolamine phosphoglyceride and stained positively for both phosphate and primary amine. TLC (chloroform-methanol-water, 65:35:4) \( R_f = 0.61 \). The infrared spectrum (Figure 31) is distinguished by bands at 2400-3300, 1630, 1550 (N-H); 1230 (PO\(_2^-\)); 1070 (PO\(_2^-\), (P)OC); 1035, 890 (C-C-N); 830, 720-800 (O-P-O). A single elemental analysis yielded the following results:

\[
\text{C}_{25}\text{H}_{50}\text{O}_7\text{NSP requires: } C 55.6, H 9.3, N 2.6, S 5.9, P 5.7\% \\
\text{found: } C 55.5, H 9.6, N 2.5, S 5.8, P 5.6\%
\]

Preparation of 1-S-hexadecanoyl-1-mercapto-2,3-propanediol (XIX) and 1-S-decanoyl-1-mercapto-2,3-propanediol (XX). The sulfhydryl group of mercaptoglycerol was selectively acylated with hexadecanoyl chloride as described by Aarsman et al. (1976) for the monacylation of mercaptoethanol. Mercaptoglycerol (100 mmol) was dissolved in 100 ml diethyl ether and 60 mmol pyridine. To this solution was added dropwise a solution of hexadecanoyl chloride (25 mmol) in 30 ml diethyl ether with rapid stirring at 0°C. After the addition, the mixture was allowed to stir at room temperature for two hours, then extracted with four
50 ml portions of water. The organic phase was dried over Na$_2$SO$_4$ and rotary evaporated. The residue was taken up in chloroform and eluted from a 200 g column of neutral silica gel with chloroform, chloroform-methanol (98:2), and chloroform-methanol (96:4). The monoglyceride (XIX) elutes in the last fraction, and the purified product was crystallized from benzene at 4° C in 30% yield (m.p. 83.5-84.5° C). It migrates slightly ahead of monopalmitin on tlc (chloroform-methanol, 96:4, $R_f$=0.55 for (XIX) compared to 0.50 for monopalmitin). The infrared spectrum exhibited the following strong bands: 3450, 1410 (O-H); 2960, 2930, 2860, 1460 (C-H); 1690 (thioester); 1060, 1030 (C-O).

1-S-decanoyl-1-mercapto-2,3-propanediol (XX) (m.p. 68.0-69.0° C) was prepared by an identical procedure from decanoyl chloride.
Results

Thioester analogs of phosphatidyl choline (XVII), phosphatidyl ethanolamine (XVIII), and monoglyceride (XIX and XX) were prepared in moderate yield from mercaptoglycerol. For all of these substrates, the essential feature of any synthetic route is that it provide the final products in high isomeric purity. For example, the 1,3-isomers of PC (XVII) and PE (XVIII) may be colorimetric substrates for phospholipase A$_2$ (van Deenen and de Haas, 1963); the enzyme specificity for phospholipase A$_1$ would therefore be lost if significant amounts of the 1,3-isomer were present in the final products. Likewise, we have shown that the thiophosphoester isomers of PC (XVII) and PE (XVIII) are substrates for phospholipase C, and these must also be avoided (Cox et al., 1979).

We have developed two synthetic routes to satisfy the isomeric purity criterion. The first, which is not reported in detail here, involves blocking the sulfhydryl group of mercaptoglycerol by oxidation to the disulfide. Subsequent tritylation and reduction of the disulfide with dithiothreitol gave 3-trityl-1-mercaptop-2,3-propanediol. This intermediate was then converted into the diglyceride (XVI) by diacylation and removal of the trityl blocking group on a boric acid/silicic acid column. This five step route to (XVI) was time consuming and the yield was low.
On the other hand, partial hydrolysis of the triglyceride of racemic mercaptoglycerol with \textit{R. delemar} lipase provides a convenient two step route to (XVI) with an overall yield of 25\% (Figure 30). The success of this approach is attributable to the large difference in polarity between the various lipolysis products that allows (XVI) to be isolated in high isomeric purity by column chromatography. The stereoisomeric composition of (XVI) depends on the stereospecificity of the lipase. This enzyme is reported to be nonstereospecific since it hydrolyzes both the 1 and 3-positions of natural triglycerides (Tsujisaka et al., 1972). We also observed the formation of the expected mixture of di- and monoglycerides upon lipolysis of (XV) (Figure 30). Thus, the diglyceride (XVI) is probably a racemate or at least only partially resolved. Both thioester and ester bonds were hydrolyzed at comparable rates, as judged by the order of appearance of products in the reaction mixture.

The phosphocholine and phosphoethanolamine derivatives of (XVI) were synthesized by published procedures for diglycerides of glycerol (Aarsman et al., 1976; Eibl, 1978). Structures of PC (XVII) and PE (XVIII) analogs were verified by tlc behavior, infrared spectra (Figure 31), and elemental analyses. The structural isomers of (XVII) and (XVIII), 1,3-S,0-didecanoyl- and 2,3-O-didecanoyl-glycerophospholipics, could not be detected by tlc, on
Figure 30. Synthetic scheme for thioester substrates for phospholipase A1 and monoglyceride lipase. R is CH$_3$(CH$_2$)$_8$- and R' is CH$_3$(CH$_2$)$_8$- for (XX) and CH$_3$(CH$_2$)$_{14}$- for (XIX).
Figure 31. Infrared spectra of rac-2,3-didecanoyl-glycero-1-phosphocholine (DDL), rac-1,2-S,O-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (XVII), and rac-1,2-S,O-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (XVIII). Spectra were obtained as 10% (W/V) solutions in chloroform against a blank cell containing chloroform.
Figure 31.
which both migrate slightly ahead of the 1,2-S,0-didecanoyl-
isomer. These thioester substrates should therefore be
specific for phospholipase A. The overall synthesis of
both PC (XVII) and PE (XVIII) as shown in Figure 30 requires
only three workups and can be easily completed in ten days.

We also prepared two thioester analogs of monogly-
cerides, 1-S-hexadecanoyl-1-mercapto-2,3-propanediol
(XIX) and 1-S-decanoyl-1-mercapto-2,3-propanediol (XX) as
monopalmitin (MP) and monodecanoin (MD) analogs, respec-
tively. Neither of the purified products contained any
oxyester bonds detectable by infrared spectroscopy, nor
were any sulfhydryl groups detected by reaction of the
dispersed substrate with DTP.

Incubation of R. delemar lipase with substrate sus-
pensions in the presence of DTP resulted in a linear
increase in absorbance at 324 nm. When 0.05 mM substrate
concentrations were used, complete hydrolysis of all the
thioester bonds could be spectrophotometrically monitored
(1.0 A unit corresponds to 50.5 nmoles of thiol). After
subtracting for changes in turbidity, measured at 450 nm
where neither DTP nor thiopyridone absorb, the net absorb-
ance change corresponded to a release of 104.7, 99.6, and
99.7% of the theoretical thiol content for PC (XVII),
PE(XVIII), and MP (XIX), respectively. Treatment of
PC (XVII) and PE (XVIII) with neither phospholipase A (Crotalus adamanteus) nor phospholipase C (Bacillus cereus)
resulted in thiol release. No discontinuities were detected in any of the progress curves to suggest enzymic stereoselectivity for the racemic substrates.

As described in Methods, it was necessary to stabilize the suspensions of substrates PE (XVIII) and MP (XIX) to prevent sedimentation of very fine crystals. If chloroform was not completely removed from PE (XVIII) prior to addition of buffer and sonication, stable emulsions with low turbidity were produced. A series of emulsions containing PE (XVIII) and various amounts of chloroform were therefore prepared to more closely examine the solvent effect. Concentrations of chloroform as low as 0.8 mM for a 0.5 mM emulsion of PE (XVIII) reduced the A$_{450}$ by 72% and increased the rate of hydrolysis by 800% (Figure 32). There was a good inverse correlation between A$_{450}$ and hydrolysis rate curves (P>95%). Both the duration of the linear phase of the progress curve and the enzyme concentration range over which linear enzyme dependencies could be obtained were increased by increasing the concentration of chloroform. At the same concentration, neither diethyl ether nor isopropanol were as effective as chloroform at producing stable emulsions of PE (XVIII). However, chloroform did not facilitate the dispersion of MP (XIX). Sodium taurocholate was satisfactory unless the pH neared the pK of the carboxyl group (pH 6), in which case the dispersions rapidly precipitated. DidecanoylGPC produced semistable suspensions
Figure 32. Effect of chloroform on the $A_{450}$ of PE(XVIII) emulsions (o) and on R. delemar lipase activity (△). Incubations were conducted at pH 7.0 with 1 mg powder (600 U/mg).
in a 1:1 molar ratio with MP (XIX), and was unaffected by pH. It was therefore selected as the dispersing agent for both MP (XIX) and MD (XX) for further studies.¹

Reaction product composition as a function of DTP concentration from the incubation of *R. delemar* lipase with PE (IV) emulsions was examined by tlc (Figure 33). The reaction progress of incubations containing 0.1 mM PE(XVIII) and varying ratios of DTP to PE (6, 1, 0.5, and 0) was followed spectrophotometrically. The three DTP containing incubations had identical progress curves even though the DTP in one of the incubations was insufficient to react with the lysoPE(XVIII) product. The other hydrolysis product, decanoic acid, co-migrates with PE(XVIII) in the chloroform-methanol-NH₄OH (65:35:4) solvent system. Thus the PE(XVIII) spot was still present

¹ Although Triton X-100 produced a stable emulsion for PE(XVIII) in a molar ratio of TX100: PE(XVIII) of two, this emulsion was inactive with our colorimetric assay. We have previously used TX100 as detergent for bovine brain ethanolamine phospholipid hydrolysis by the lipase and saw neither a marked stimulation nor inhibition by a broad range of TX100: PE ratios when assayed by a tlc separation-phosphorus analysis (Cox, 1977). These results suggest that either TX100 interferes with our assay method, or that the inhibition is specific for shorter chain ethanolamine phospholipids. Likewise, during the development of a phospholipase C assay using thiophosphoester substrates and *B. cereus* phospholipase C, we observed substrate inhibition for a 2:1 TX100:PE substrate emulsion. The inhibition became noticeable at 10 μM, which is the cmc of the PE substrate. The enzyme activity completely disappeared at 30 μM substrate. Roberts et al. (1978) have studied the substrate specificity of *B. cereus* phospholipase C with TX100 substrate emulsions, and did not observe substrate inhibition. The reason for the aberrant TX100 results in our assay system is unclear.
Figure 33. TLC of reaction products from incubation of PE(XVIII) with R. delemar lipase at different concentrations of DTP. Solvent system: chloroform-methanol-ammonia (65:35:4). Standards are decanoic acid (DA) and PE(XVIII). Hatch marks represent a positive stain for phosphorus. Incubations contained 0.1 mM PE(XVIII) at pH 6.0.
after the incubation, but it no longer stained positively for phosphorus. The lysoPE product formed in the absence of DTP was not extracted into the organic phase, but two new phosphorus containing spots appeared in the extracts from DTP containing incubations. The less polar of the pair was the major product in the presence of high DTP concentrations and this material is presumably the mixed disulfide of lysoPE (XVIII) and thiopyridine. The more polar spot predominates as the DTP concentration decreases, and it is probably the symmetrical disulfide of lysoPE (XVIII). Unreacted DTP migrates to the solvent front.

Linear enzyme dependencies were obtained for each substrate over a minimum 10 fold range of protein concentration (Figure 34). The slopes of these plots were unaffected by altering the DTP concentration, but reducing its concentration reduced the protein concentration range over which a first order dependency was obtained.

The modulatory effect of Ca$^{2+}$ for the three substrates at pH 6.0 was examined (Figure 35). Whilst Ca$^{2+}$ moderately stimulated the initial rate of hydrolysis with sodium taurocholate-MP(XIX) dispersions and had little effect on the hydrolysis of didecanoylGPC-MP(XIX) dispersions, it inhibited the hydrolysis of both PC(XVII) and PE(XVIII). The inhibitory effect with PE(XVIII) is at least partly due to the destabilization of the PE emulsion and the precipitation of the substrate. However, inhibition of
Figure 34. Enzyme dependency curves for the reaction of *R. delemar* lipase with PC(XVII), PE(XVIII), and MP(XIX) at pH 7.0. Incubations contained 0.6 mM DTP.
Figure 35. Effect of Ca\(^{2+}\) on the activity of \textit{R. delemar} lipase with thioester substrates. MP(XIX) was dispersed with 1:1 molar ratios of either sodium taurocholate or di-decanoyl lecithin. Incubations contained 0.5 mM PC(XVII) and PE(XVIII), and 0.2 mM MP(XIX). PE(XVIII) dependence is indicated with a dashed line since PE emulsions containing CaCl\(_2\) were unstable.
PC(XVII) hydrolysis was not associated with a visible (A450) substrate perturbation. Stimulation of MP(XIX) hydrolysis is apparently due to the ability of Ca2+ to prevent product inhibition (Figure 36). In the absence of Ca2+, the hydrolysis rate rapidly decreases from zero time. If Ca2+ is added any time during the incubation, however, the hydrolysis accelerates to reach the control value containing Ca2+. A similar study with PC (XVII) failed to demonstrate any ability of Ca2+ to stimulate hydrolysis. Ca2+ stimulation was also observed with MD (XX) so that the opposite results with PC(XVII) and MP (XIX) are not attributable to the difference in fatty acid chain length between the substrates.

The pH dependencies for the hydrolysis of MP(XIX), PE(XVIII), and PC(XVII) by the lipase are given in Figure 37. PE(XVIII) emulsions were unstable below pH 4.3. However, there was no significant change in A450 for the pH 4.3 to 7.5 emulsions for all three substrates. Linear enzyme dependencies were obtained with PE(XVIII) at each pH, indicating that the thiol detection system is still responsive under mildly acidic conditions. The pH optima for the hydrolysis of MP(XIX) and PE(XVII) were similar at pH 6.0 and 5.7 respectively, but the pH optimum for PC(XVII) was less than 4.5. At pH 6.0 the PE(XVIII) hydrolyzing activity was only 3% and that for PC(XVII)
Figure 36. Ca$^{2+}$ stimulation of MP(XIX) hydrolysis by R. delemar lipase. Curve (A) is with 5 mM CaCl$_2$, curve (B) is with 1 mM EDTA. Arrows indicate the addition of 5 mM CaCl$_2$ to curve (B).
Figure 37. pH dependency curves for the hydrolysis of MP(XIX), PE(XVIII), and PC(XVII) by R. delemar lipase (600 U/mg). Rates are expressed on the vertical axes as umol/h/mg powder. MP(XIX), (A); PE(XVIII), (o); PC(XVII), (□).
0.3% of the activity with MP(XIX). MD(XX) was hydrolyzed 75% as fast as MP(XIX).

The lipase and phospholipase activities were also different in heat stability (Figure 38). Although both showed activation by heating up to 48°C, the monoglyceride lipase activity continued to increase up to 56°C while the phospholipase activity declined over the same period. A ratio analysis of the enzyme activities at pH 6.0 and 4.5 after preincubation of the enzyme at various temperatures revealed a difference in heat stability between lipase and the phospholipase activities (Table 7).

Whereas the ratio of the MP(XIX) hydrolyzing activities at pH 6.0 and 4.5 is unchanged by heating, the ratios for both PC(XVII) and PE(XVIII) are altered. In general, the PE(XVIII) hydrolyzing activity is less stable than either the PC(XVII) or MP(XIX) activities. Both the PC(XVII) and PE(XVIII) activities at pH 4.5 are less stable than the same activities at pH 6; this is especially true of PE(XVIII).

If several lypolytic activities are present, then it is possible that the relative amounts of activity for each substrate will be different in the purified grade of _R. delemar_ lipase from Miles Laboratories. The ratios of activities for the purified and crude preparations at both pH 4.5 and 6 are given in Table 8. The purified preparation is 7-8 times as active as the crude preparation for each
Figure 38. Heat stability curves for *R. delemar* lipase. Enzyme solutions were heated at the indicated temperature for 10 min, pH 6, then cooled in ice. Assays with MP(XIX), PE(XVIII), and PC(XVII) were conducted at 25°C at both pH 6.0 and 4.5.
Figure 38.

- PE(XVIII) pH 6.0 ○
- PE(XVIII) pH 4.5 ●
- PC(XVII) pH 6.0 △
- PC(XVII) pH 4.5 ▽
- MP(XIX) pH 6.0 □
- MP(XIX) pH 4.5 ▼
Table 7. Effect of heat pretreatment on the ratio of lipase activities as assayed with thioester substrates at pH 4.5 and 6.0. Ratios are calculated as the ratio of enzyme specific activities.

<table>
<thead>
<tr>
<th>temperature (°C)</th>
<th>PE 6.0 / PE 4.5</th>
<th>PC 6.0 / PC 4.5</th>
<th>MP 6.0 / MP 4.5</th>
<th>PE 6.0 / PE 4.5</th>
<th>PE 4.5 / PE 4.5</th>
<th>MP 6.0 / MP 4.5</th>
<th>MP 4.5 / MP 4.5</th>
<th>MP 6.0 / MP 4.5</th>
<th>MP 4.5 / MP 4.5</th>
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<tr>
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<td>2.57</td>
<td>5.68</td>
<td>0.60</td>
<td>26.7</td>
<td>30.8</td>
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<td>18.6</td>
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<tr>
<td>40</td>
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<td>0.33</td>
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<td>6.20</td>
<td>0.63</td>
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<td>33.6</td>
<td>168</td>
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</tr>
<tr>
<td>48</td>
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<td>0.32</td>
<td>2.74</td>
<td>6.82</td>
<td>0.58</td>
<td>22.9</td>
<td>31.8</td>
<td>156</td>
<td>18.3</td>
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<tr>
<td>56</td>
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<td>6.04</td>
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<tr>
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<td>1.47</td>
<td>0.14</td>
<td>79.0</td>
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<td>117</td>
<td>34.5</td>
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</tbody>
</table>
Table 8. Comparison of lipolytic activities of crude (600 U/mg) and purified (6000 U/mg) *R. delemar* lipase as assayed with thioester substrates at pH 4.5 and 6.0.a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>Purified/crude activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>6.0</td>
<td>7.5</td>
</tr>
<tr>
<td>PE</td>
<td>4.5</td>
<td>7.4</td>
</tr>
<tr>
<td>PC</td>
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<tr>
<td>MP</td>
<td>6.0</td>
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</tr>
<tr>
<td>MP</td>
<td>4.5</td>
<td>7.2</td>
</tr>
</tbody>
</table>

a. Specific activities and activity unit definition are as described by Miles Laboratories (Elkhart, IN)
Figure 39. pH dependency curves for the hydrolysis of PE(XVIII) (○) and PC(XVII) (△) by the purified preparation of R. delebrar lipase (6000 U/mg). Rates are expressed as umol/h/mg powder on the vertical axes.
Figure 40. pH dependency curves for the hydrolysis of PE(XVIII) (○) and PC(XVII) (△) by R. arrhizus lipase (6000 U/mg). Rates are expressed as μmol/h/mg powder on the vertical axes.
substrate at pH 4.5 and 6, with the exception of PC(XVII) at pH 6. These experiments were repeated twice with the same results. Both enzyme preparations were assayed with the same substrate suspensions, so that differences in the quality of substrate dispersion cannot account for the observed change in specific activity. The relatively greater PC(XVII) activity with the purified preparation at pH 6 is manifested as a broader pH optimum, with a shoulder extending to pH 6 before beginning a rapid decline (Figure 39).

*Rhizopus arrhizus* and porcine pancreatic lipases have been reported to possess both phospholipase A₁ and triglyceride lipase activities. We examined the pH optima for the hydrolysis of PE(XVIII) and PC(XVII) by a commercial *R. arrhizus* preparation (Boehringer Mannheim). It closely resembled the pH dependency observed with *R. delemar* lipase (Figure 40). PC(XVII) hydrolysis had two optima: a major peak from pH 4 to 5 and a minor peak coinciding with PE(XVIII) hydrolysis at pH 6.0. The triglyceride lipase pH optimum is 7.0 (Labourer and Labrousse, 1966).
Discussion

Because of the broad specificity of commercial R. delemar lipase for both triglycerides (Tsujisaka et al., 1972) and phospholipids (Cox, 1977), the same enzyme preparation could be used to synthesize thioester substrates for phospholipase A₁ and to develop the spectrophotometric phospholipase A₁ assay. The synthesis of isomerically pure 1-S-2-O-didecanoyl-1-mercapto-2,3-propanediol requires a method for protecting the 3-hydroxyl group. The use of hydroxyl blocking groups in the organic synthesis of glycerolipids is common and usually necessary to obtain the desired level of isomeric or stereoisomeric purity. For example, an often used synthetic route to monoacid diglycerides is to protect the hydroxyl group of allyl alcohol with dihydropyran prior to oxidizing the vinyl group with permanganate (Jensen and Pitas, 1976). The resulting pyranylated glycerol can then be acylated and the pyran group removed with boric acid. For a large scale synthesis, this four step procedure is satisfactory. On the other hand, for lipid preparations of less than 10 g, it is more convenient to partially hydrolyze a monoacid triglyceride with lipase.

We initially tried a five step procedure to obtain the 1,2-diglyceride of mercaptoglycerol involving two blocking and deblocking steps. This scheme gave no better isomeric purity and lower yields than a procedure employing
a lipolysis step. The expected glyceride mixture was obtained upon treatment of the triglyceride of mercapto-glycerol with \textit{R. delemar} lipase, but the desired diglyceride product could be isolated in high purity in 26\% yield. Judging from the product distribution on tlc, it was estimated that the lipase hydrolyzed thioesters and esters at similar rates.\textsuperscript{2}

The infrared spectrum of the 1,2-diglyceride exhibited strong hydroxyl, ester, and thioester bands. The same ester thioester absorbance spectrum was conserved in the phosphocholine and phosphoethanolamine derivatives (Figure 31). TLC behavior and elemental analyses confirmed the structures of PC(XVII) and PE(XVIII). When hydrolyzed by \textit{Bacillus cereus} phospholipase C or \textit{Crotalus adamanteus} phospholipase A\textsubscript{2}, there was no increase in absorbance corresponding to the release of thiols. Treatment with \textit{R. delemar} lipase resulted in an absorbance change for total hydrolysis of the substrate corresponding to the theoretical amount of thioester present (assuming a thioester: phosphorus ratio of 1.0). Since the \textit{R. delemar} lipase

\textsuperscript{2} Another product of the reaction is the 2,3-diglyceride possessing a free sulfhydryl group. We have previously reported the synthesis of this compound as an intermediate in the preparation of thiophosphoester substrates for phospholipase C (Cox et al., submitted). The synthetic procedure involved oxidation of mercaptoglycerol to the disulfide followed by tetraacylation and reduction. This route is still considered superior to partial lipolysis because it proceeds in high yield and requires only one purification step.
phospholipase activity consists only of phospholipase \( A_1 \) and lesser amounts of lysophospholipase (Cox, 1977), these thioester substrates are specific for phospholipase \( A_1 \). In heterogenous enzyme systems, thiol release could also result from the combined action of a phospholipase \( A_2 \) and lysophospholipase or of a phospholipase \( C \) and a diglyceride lipase. The same possibilities exist with radiochemical assays, however, and both types of assays require product analysis to verify the identity of the hydrolyzing activity.

The slope of the recorder tracing for increasing absorbance due to thioester hydrolysis depended on both substrate and enzyme concentrations. There was a clearing effect during the hydrolysis, i.e., a decrease in turbidity resulting from conversion of the substrate into a more water soluble form. The clearing slowly developed with time and was never a factor in initial rate measurements.

The products were analyzed by tlc to verify the formation of fatty acid and to ascertain if the product identity was dependent on DTP. For water insoluble thiols, disulfide exchange to form symmetrical disulfides at the micelle surface predominates regardless of the DTP concentration (Cox et al., 1979). The results from our experiments with phospholipase \( A_1 \) substrates were those expected of a homogenous system; there did not appear to be any surface effect. A phosphorus containing material
with the expected $R_f$ of the disulfide formed from lysoPE and DTP was the predominate product at high DTP concentrations (Figure 33). Low concentrations of DTP resulted in the formation of a phosphorus containing material with the polarity expected of the symmetrical lysoPE(XVIII) disulfide. The shape of the progress curve was not influenced by changing the DTP/PE ratio from 6 to 0.5.

Linear enzyme dependencies were obtained for each substrate, and for PE(XVIII) over the pH range 4.5-7.5. DTP is reported to give theoretical values for thiol content over the pH range 3-8, thus allowing assays to be performed in acidic media. Identical rates were obtained at pH 7 using either DTP or DTNB, and altering the concentration of the thiol reagent had no effect on the slope of the enzyme dependency curve.

A monopalmitin thioester analog (MP(XIX)) was synthesized in order to compare the monoglyceride lipase and phospholipase activities of \textit{R. delemar} lipase. With sodium taurocholate as the dispersing agent, at pH 7 MP(XIX) was hydrolyzed 30 and 300 times faster than PE(XVIII) and PC(XVII) respectively. The relative inertness of didecanoylglycerol allows it to be used as neutral dispersing agent for MP(XIX).

The monoglyceride lipase and phospholipase activities differed with respect to $Ca^{2+}$ stimulation, pH optima, and heat stability. As measured with MP(XIX), the monoglyceride
lipase activity is similar to the triglyceride lipase activity, and the two probably represent the same enzyme. The pH optimum for MP(XIX) hydrolysis is 6.0, compared to the expected value of 5.6 for triglyceride hydrolysis (Iwai and Tsujisaka, 1974), and the Ca$^{2+}$ stimulation patterns for the hydrolysis of both substrates are identical (Tsujisaka et al., 1972). Ca$^{2+}$ exerts little effect on the initial rate of hydrolysis, but acts to prevent enzyme inhibition during the course of substrate hydrolysis. In contrast, PC(XVII) hydrolysis was inhibited by Ca$^{2+}$ whether it was added initially or after 50% hydrolysis. Since MP(XIX) is dispersed in didecanoylGPC, and Ca$^{2+}$ causes no change in turbidity for either MP(XIX) or PC(XVII) emulsions, this difference in behavior is probably not related to a Ca$^{2+}$ induced change in the physical state of either substrate. Unlike PC(XVII), PE(XVIII) was precipitated by Ca$^{2+}$, making comparisons of Ca$^{2+}$ modulation impossible.

The PC(XVII) phospholipase A$_1$ and MP(XIX) monoglyceride lipase activities were also different in pH optima. Whereas the optimum for PE(XVIII) was similar to MP(XIX) (5.6 and 6.0, respectively) and in agreement with the reported value for triglyceride lipase, the optimum for PC(XVII) hydrolysis was less than 4.5. Furthermore, the MP(XIX) monoglyceride lipase activity is more heat stable than either of the phospholipase activities. Differences in the heat stability of PC(XVII) and PE(XVIII) hydrolyzing activities suggest the
presence of several lipolytic activities with different substrate specificities.

A preparation of *R. delemar* lipase advertised as having ten fold higher specific activity of the triglyceride lipase than the crude preparation was examined for phospholipase activity. MP(XIX), PC(XVII), and PE(XVIII) hydrolyzing activities at pH 4.5 and 6.0 all had 7-8 fold higher specific activities, with the exception of a ten fold increase in PC(XVII) specific activity at pH 6.0. The pH curve for PE(XVIII) hydrolysis was the same in both crude and purified preparations. The PC(XVII) curve had a broader optimum extending to pH 6 in the purified lipase.

*Rhizopus arrhizus* lipase has been purified and shown to hydrolyze phospholipids (Slotboom et al., 1970). A commercial powder of the lipase had about the same proportion of PE(XVIII) and PC(XVII) phospholipase activities as *R. delemar* lipase, and the pH optimum for PE(XVIII) hydrolysis was the same in both preparations. PC(XVII) hydrolysis had an optimum at pH 5.0 and a definite shoulder at 6.0.

From this evidence, it appears that *R. delemar* lipase, and probably *R. arrhizus* lipase, contain a PC specific phospholipase activity. The PC(XVII) hydrolyzing activity represents only 0.3% of the MP(XIX) hydrolyzing activity at pH 6.0 and 1% at pH 4.5. It may therefore be due to a minor lipase in the mixture that has an acidic pH optimum and a broader substrate specificity, or it could represent
a trace amount of true phospholipase A\textsubscript{1}. On the basis of heat stability, the same could be said of the PE(XVIII) hydrolyzing activity.

The progress curves for the hydrolysis of MP(XIX), PC(XVII), and PE(XVIII) did not display the characteristic shape for an enzyme that preferentially hydrolyzed the L-isomer of a racemic mixture. Some investigators have suggested that the presence or absence of stereospecificity should be used as criterion to distinguish phospholipase A\textsubscript{1} from triglyceride lipase (Brockerhoff and Jensen, 1974; Scandella and Kornberg, 1971). Although all triglyceride lipases examined thus far are nonstereospecific (Tsujisaka et al., 1972; Brockerhoff and Jensen, 1974), it is premature to classify an enzyme as a triglyceride lipase on the basis of its ability to hydrolyze D-lecithin without more evidence on the steric requirements of several phospholipases A\textsubscript{1}.

We have reported a simple three step procedure for the preparation of thioester substrates for phospholipase A\textsubscript{1}. The assay is sensitive (10 nmol/h corresponds to 0.2 A/h with DTP), specific for phospholipase A\textsubscript{1}, and useful over the pH range 3-8 with DTP and 7-8.5 with DTNB. The possibility of separating the triglyceride lipase and phospholipase A\textsubscript{1} activities of \textit{R. delemar} lipase is currently under investigation.
CHAPTER VI

PARTIAL SYNTHESIS OF A THIOPLASMALOGEN.

STEREOSPECIFIC SYNTHESIS OF CIS-ALKENYL
THIOETHERS OF MERCAPTOGLYCEROL

Introduction

Plasmalogenase is the enzyme that hydrolyzes the enolether linkage of plasmalogens (lipids containing the alk-1'-enylglycerol moiety) to produce fatty aldehyde and lysophospholipid (Ansell and Spanner, 1965). It is activated in brain white matter during the early stages of canine distemper and is postulated to be an initiator of demyelination in the central nervous system (Horrocks et al., 1978). A rapid and convenient enzyme assay would be of clinical importance.

We have previously described the use of sulfur substituted analogs of phospholipids as spectrophotometric substrates for phospholipases A_1 and C (Cox et al., 1979; Cox et al., submitted). These analogs contain thioesters and thiophosphoester bonds, respectively, and when hydrolyzed by the appropriate enzyme release thiols that react with colorimetric thiol reagents. The first assay of this type was reported for acetylcholinesterase (Ellman et al.,
1961), and Aarsman has applied the technique to the assay of phospholipase A\textsubscript{2} (Aarsman and van den Bosch, 1977; Aarsman et al., 1976). Similarly, a sulfur analog of plasmalogen might provide a convenient assay for plasmalogenase.

The organic synthesis of plasmalogens is a difficult problem (Gigg, 1972; Paltauf, 1973). Besides the presence of two centers for isomerism, the asymmetric C-2 carbon of glycerol and the cis-double bond of the enolether linkage, the acid lability of the enolether group severely restricts the types of reactions that can be employed. The same problems apply to the synthesis of a thioplasmalogen containing an enethiolether linkage.

Hirsch (1976) prepared a cis/trans mixture of the 1-heptenyl thioether of mercaptoglycerol by reaction of the Li/NH\textsubscript{3}(liq) reduction product of 1-ethylthioheptene with 1-bromo-2,3-propanediol. Because of the vast array of reactions in sulfur chemistry, it seemed probable that an alternative route could be devised that would yield only the cis-isomer. We decided to take advantage of the configurational stability of cis-1-alkenyl lithium compounds (Wakefield, 1974) and their reaction with disulfides to produce cis-1-S-dec-1'-enyl-2,3-isopropylidene-1-mercapto-2,3-propanediol (XXIV). The procedure can be altered to yield the optically active product if so desired.
Materials and Methods

Materials. These chemicals were purchased from the following companies: n-BuLi (1.6 M in hexane), and catecholborane (Aldrich Chemical Company, Milwaukee, Wisconsin); mercaptoglycerol (Evans Chemetics, Darien, Connecticut); thiolacetic acid (Eastman Organic Chemicals, Rochester, New York); and Li metal (1/8 inch wire containing 1% sodium) (Ventron Corporation, Beverly, Massachusetts). Tetrahydrofuran (THF) and diethyl ether were refluxed for 2 hours over Na chips prior to distillation. Methylene chloride (CH₂Cl₂) was dried over CaSO₄, distilled, and collected over molecular sieve.

Analytical. Infrared spectroscopy was performed on a Beckman Model 4230 IR Spectrophotometer, NMR was done with a Varian T 60, and GC/MS was performed on a Hewlitt Packard HP 5985. Analytical GLC was done with a Varian Aerograph Model 920 Gas Chromatograph equipped with a column of 15% ethylene glycol succinate on Gas Chrom P, 80-100 mesh. Gas flow rate was 60 ml/min and all runs were isothermal.

Preparation of 1,1'-dithiobis(2,3-isopropylidene-2,3-propanediol)(XXI). Glycerol (1 mol) was dissolved in 2 mol acetone and 700 ml benzene. p-Toluenesulfonic acid (2 g) was added and the solution refluxed overnight with a Dean Stark trap. After rotary evaporation of the solution to a viscous oil, 400 ml pyridine and 0.95 mol
p-toluenesulfonyl chloride were added. The mixture was stirred for two days and then added to 21 diethyl ether and the resulting mixture extracted twice with one l portions of water, 500 ml 5% NaHCO₃ (aq), and 500 ml saturated NaCl (aq). The ether phase was filtered through Na₂SO₄, dried over CaSO₄, filtered and rotary evaporated to give a yellow oil that solidified on standing (262 g).

Thiolacetic acid (1.32 mol) was added dropwise to a 40% solution of KOH in methanol (1.32 mol KOH) cooled in an ice bath. Solvent was rotary evaporated with several additions of benzene to remove water. The residue was taken up in 1.5 l ethanol and heated to reflux. The tosylated isopropylidene glycerol (1.0 mol) was added and the solution refluxed for 1 hour. It was then cooled in an ice bath and suction filtered through a Buchner funnel. The filtrate was rotary evaporated and taken up in 400 ml water. A reddish oil separated out as a lower phase and was drained off. The aqueous phase was washed three times with 200 ml diethyl ether, and the combined organic phases were extracted with 150 ml saturated NaCl (aq). The ether was rotary evaporated from a one round bottomed flask and 400 ml 20% NaOH (aq) added for overnight thioester hydrolysis. The aqueous solution was then extracted with 200 ml diethyl ether to remove any material insoluble in aqueous base, and the aqueous phase was sampled for sulfhydryl group determination with 5,5'-dithiobis
(2-nitrobenzoic acid) (DTNB). It was found to contain 0.54 mol sulfhydryl groups. I₂ (0.27 mol) was slowly added (the reaction is mildly exothermic) and the remaining sulfhydryl content assayed as 0.2 mol. More I₂ (0.1 mol) was added. A dark brown oil separated during the reaction and was collected by draining the lower aqueous phase off through a separatory funnel. Distillation of the oil through a 10 cm vacuum jacketed Vigreaux column (122° C, 0.2 mm Hg) gave 46.3 g of a pale yellow oil (XXI) (31% yield). The infrared spectrum of (XXI) was identical to isopropylidene glycerol except for the absence of hydroxyl absorption bands. IR (2860-2970, 1460 (weak), 1375, C-H; 1250, 1220, 1150, 1050, isopropylidene group). The molecular weight was estimated at 303 on the basis of sulfhydryl groups liberated by Zn²⁺/HCl reduction.

Preparation of cis-1-decenyl bromide (XXII). This synthesis was performed as described by Brown (1975) for the synthesis of cis-1-octenyl bromide. 1-Decyne (0.112 mol) was introduced via a syringe to a flame dried (under N₂) three neck flask fitted with a rubber stopple and a gas inlet connected by a T tube to a N₂ source and a mineral oil bubbler. Catecholborane (0.112 mol) was added and the mixture stirred for 2 hours at 70° C. After cooling to room temperature, dry CH₂Cl₂ (125 ml) was introduced with a double ended needle and the temperature reduced to -10° C. Br₂ (0.190 mol) was then added dropwise with rapid
stirring so that the temperature never exceeded \(-5^\circ C\).

After stirring at \(-10^\circ C\) for 45 minutes, 200 ml 2M sodium methoxide (prepared from sodium metal and methanol) was added so that the temperature never exceeded \(0^\circ C\). The mixture was stirred for 1 hour and then warmed to room temperature. Water (100 ml) was added and the mixture extracted. The aqueous phase was washed with two 25 ml portions of CH\(_2\)Cl\(_2\) and the organic phases combined. A dark oil formed at this point that could be removed by reextraction with 50 ml water. The organic phase was filtered through Na\(_2\)SO\(_4\), dried over CaSO\(_4\), and vacuum distilled through a 10 cm Vigreaux column. The distillate was collected in four fractions (0.45 mM Hg, 60.5-61.5, 61.5-63.0, 63.0-65.0, and 65.0-67.0\(^\circ\) C) and analyzed for purity by GLC. Fractions 2, 3, and 4 were combined to give the product (XXII) 96% pure by triangulation (GLC \(R_t=3.6\) minutes, 145\(^\circ\) C). IR(3040, 1625, 700-650, cis-vinyl bromide; 2860-2970, 1470, C-H; 1300, unidentified). NMR (CDC\(_3\)/TMS) \(5.8-6.2(m,2)\), \(1.9-2.4(m,2)\), \(1.25(s,12)\), \(0.8(t,3)\). Mass spectrum m/e (relative intensity) 218 (1,\(M^+\)), 220 (1,\(M^++2\)). The remaining 4% of the material has a GLC \(R_t\) of 2.7 minutes, possesses a carbonyl group (1740 cm\(^-1\)), and is probably decanal.

Preparation of l-S-dec-1'-ynyl-2,3-isopropylidene-1-mercapto-2,3-propanediol (XXIII). 1-Decyne (26 mmol) was dissolved in 15 ml dry THF in a flame dried, Ar atmosphere,
three neck flask equipped with a gas inlet, dropping funnel, and rubber stopple. After cooling to -10°C, a solution of n-BuLi (16.3 ml of 1.6 M n-BuLi) in 30 ml THF was added dropwise to the decyne solution so that the temperature never exceeded -5°C. It was allowed to stir at room temperature for 1 hour, and then cooled to 0°C. Disulfide (XXI) (21 mmol) was added dropwise with rapid stirring at less than 5°C and then warmed to room temperature and allowed to stir for 2 hours. Ice was added to stop the reaction and 200 ml hexane was added. The mixture was extracted with 75 ml water and 75 ml saturated NaCl (aq). It was filtered through Na₂SO₄ and dried over CaSO₄. Rotary evaporation gave a pale yellow oil which was purified on a 200 g column of silicic acid. Solvent system: hexane-diethyl ether (98:2) and (95:5). The desired product (XXIII) eluted in the second fraction in 75% yield. TLC (hexane-diethyl ether, 90:10) Rᵣ=0.70. GLC(190°C) Rₜ=27 minutes. IR(2840-2960, 1470, 1375, C-H; 1250, 1220, 1150, 1060, isopropylidene group). NMR (CDCl₃/TMS) 3.7-4.6 (m,3), 2.8 (t,2), 2.2 (m,2) 1.4 (d,15), 0.9 (t,3). Mass spectrum m/e (relative intensity) 284 (15, M⁺), 285 (4, M⁺+1).

Preparation of cis-1-S-dec-1'-eny-2,3-isopropylidene-1-mercapto-2,3-propanediol (XXIV) using Li metal. A three neck flask was fitted with a glass stopper and two condensers, one of which was capped with a gas inlet and the
second with a rubber stopple. After flame drying under an Ar atmosphere, 50 ml dry diethyl ether was introduced with a double ended needle. Freshly cut Li metal (0.10 mol cut into fine pieces under diethyl ether solvent) was then dropped into the Ar atmosphere. cis-1-Decenyl bromide (XXII) (0.05 mol) was added dropwise with a syringe over a 2 hour period while refluxing. Not all the Li reacted. The flask was then cooled to 0° C and the disulfide (XXI) added over a 15 minute period. It was stirred for 30 minutes and then warmed to room temperature and poured into 100 ml hexane. Ice was added to decompose the remaining Li metal and the mixture extracted three times with 50 ml water. The organic phase was dried over Na₂SO₄ and CaSO₄ and then rotary evaporated to give 15 g of yellow oil. GLC analysis indicated the composition of the thioether products as 75% cis-(XXIV) (Rₜ=21 minutes), 7% trans-(XXIV) (Rₜ=23 minutes), and 18% (XXIII) (Rₜ=26 minutes) at a column temperature of 190° C. TLC(hexane-diethyl ether, 90:10) R₉(XXIII)=0.7, R₉(XXIV)=0.6. The oil (4 g) was applied to 200 g columns of silicic acid and eluted with hexane-diethyl ether (98:2) to obtain 1 gm of material enriched in (XXIV). This material was pooled and reapplied to a second column to give 3.5 g of (XXIV) in 25% yield. GLC (190° C) 93.9% cis-(XXIV), 5.4% trans-(XXIV), and 0.7% (XXIII). IR(2860-2970, 1375, 1460, C-H; 1610, 1340, cis-ethiolether; 1250, 1220, 1150, 1060, isopropylidene group). Mass
spectrum m/e (relative intensity) 286 (4, M⁺), 287 (1, M⁺+1).

Preparation of cis-1-S-dec-1'-enyl-2,3-isopropylidene-
1-mercapto-2,3-propanediol (XXIV) using n-BuLi. cis-1-
Decenyl bromide (XXII) (10 mmol) was placed in a flame
dried, Ar atmosphere, three neck flask equipped with a
gas inlet and rubber stopple. Dry THF (20 ml) was
transferred into the flask with a double ended needle and
cooled to -70° C. While rapidly stirring, 5.95 ml n-BuLi
(1.6 M) was added dropwise over a 20 minute period.
Stirring was continued for 1 hour at -70° C and the
disulfide (XXI) (10 mmol) added dropwise. After stirring
for an additional hour at -70° C, the flask was warmed to
room temperature and the contents transferred to a
separatory funnel with 100 ml hexane. After washing with
three 50 ml portions of water, the organic phase was dried
over Na₂SO₄, CaSO₄, and rotary evaporated to give 4 g of
yellow oil. TLC analysis indicated a 2:1 ratio of (XXIII)
to (XXIV), and this was confirmed by GLC.
Results and Discussion

The first step in this synthesis was the preparation of the symmetrical disulfide of isopropylidene mercaptoglycerol (XXI). Previous work indicated that (XXI) was directly obtainable by acetonating mercaptoglycerol disulfide (Cox et al., submitted). However, the disulfide (XXI) is an intermediate in a reaction pathway proposed to provide optically active mercaptoglycerol, the development of which was warranted by successful assays of other phospholipases with thioester substrates (Cox et al., submitted; Cox et al., 1977; Aarsman et al., 1976; Aarsman and van den Bosch, 1976). Periodate oxidation of isopropylidene derivatives of D-mannitol (Baer, 1952) or L-arabinose (Baer and Fisher, 1939) yields 1,2- or 2,3-isopropylidene-sn-glycerols, respectively. Chapman and Owen (1950) have described a method to prepare primary thiols by thiolacetate displacement of p-toluenesulfonyl or methanesulfonyl leaving groups. Thus, we prepared the tosylate of rac-1,2-isopropylideneglycerol as described by Sowden and Fisher (1942) and displaced the tosyl group with thiolacetate in refluxing ethanol (Figure 41). Rather than work up the product at this stage (it had a strong stench), the acetate thioester was hydrolyzed in strong base to give the sodium thiolate salt of rac-1-mercapto-2,3-isopropylidene-2,3-propanediol. Unreacted material was removed at this point by ether extraction, and since the
Figure 41. Synthetic scheme for acetonated mercaptoglycerol disulfide (XXI).

b.p. 145-147 (1.3 mmHg)
yield: 30%

yield: 30%
conditions were already alkaline, the disulfide was formed
**in situ** by I₂ oxidation (Vogel, 1956). The product
separated as an insoluble oil that was collected and dis­
tilled in 31% overall yield. Starting from racemic gly­
cerol, a pair of diastereomers of (XXI) is formed, and this
may account for the broad boiling point. sn-1-Mercapto or
sn-3-mercaptoglycerols can be prepared by starting from
2,3- or 1,2-isopropylidene-sn-glycerol. After the
completion of this work, a full paper describing the
synthesis of optically active mercaptoglycerol using the
same synthetic route was communicated by Gronowitz et al.
(1978).

Because of the low percentage of side reactions during the
reaction of alkynyl lithium compounds with disulfides
to form alkynyl thioethers (Nooi and Arens, 1961), we
initially made compound (XXIII) in the hope that it could
be stereospecifically reduced to the **cis**-alkenyl thioether
(XXIV). The sulfide linkage would probably poison a
noble metal catalyst, so we attempted another stereo-
specific approach involving monohydroboration followed
by protonolysis. When the alkynyl thioether (XXIII)
was reacted successively with disiamylborane and acetic
acid as described by Brown (1975) for the reduction of
alkynes to **cis**-alkenes, several products were formed.
Preparative tlc and infrared analysis indicated the
presence of about 20% of unreacted starting material and
about an equal amount of a compound that migrated immediate-
ly behind the alkynyl thioether, stained more intensely with
I₂ vapor, and contained a double bond (1610 cm⁻¹). This
material was later confirmed to be the alkenyl thioether
(XXIV).

A cleaner reaction yielding the alkenyl thioether
(XXIV) as the main product was the reaction of cis-1-
decenyl lithium with the disulfide (XXI). As reported by
Curtin and Crump (1958), vinyl bromides can be reduced with
lithium metal in refluxing ether without substantial iso-
merization of the double bond. Starting with decenyl
bromide that was greater than 99% cis, the alkenyl
thioether was assayed in the crude reaction mixture as
91% cis. Some of the alkynyl thioether (XXIII) was also
formed, presumably by reaction of the decenyl lithium
with unreacted decenyl bromide causing elimination of
HBr to form the alkyne. Abstraction of the acidic
alkynyl proton by a second decenyl lithium molecule
yielded the alkynyl lithium intermediate. Thus, a maximum
of 3 mol of decenyl lithium is lost in the formation of
1 mol of alkynyl lithium. To make matters worse, the
two products are so similar that separation requires
careful and repeated column chromatography. After two
columns of silicic acid, the cis-alkenyl thioether (XXIV)
was isolated in about 25% yield and contained 0.7% alkynyl
thioether (XXIII) and 5% trans-isomer (Figure 42).
Figure 42. Synthetic scheme for alkenyl thioethers of mercaptoglycerol.
The ability of simple column chromatography to separate these compounds, which are structurally quite similar, is probably due to the position of the unsaturation and its contribution to the overall polarity of the molecule. These compounds possess only two functional groups that allow absorption to silica gel. These are the isopropylidene group and the unsaturated thioether linkage. The position of the unsaturation is such that it may affect interaction of the chromatography support with the isopropylidene group. Thus, there is even some resolution of the cis and trans-isomers after two silicic acid columns. Separation of alkynyl and alkenyl thioethers may result from the different resonance contribution of the sulfur in alkynyl compared to alkenyl thioethers (Silverstein et al., 1974). Once the isopropylidene group is removed, the polarity of the glycol group is likely to dwarf any contribution from the unsaturated thioether linkage and prevent separation of alkynyl (XXIII) and alkenyl (XXIV) thioether.

An alternative route to lithium metal reduction of decenyl bromide to provide decenyl lithium is metal halogen exchange. Vinyl bromides are reported to undergo exchange with alkyl lithium compounds at low temperature with retention of steric configuration (Wakefield, 1974). THF solvent favors the exchange reaction over elimination. However, reaction of cis-1-decenyl bromide with n-BuLi
in THF at -70° C followed by reaction with disulfide (XXI) gave the alkynyl thioether as the major product. In this system, elimination is the predominant reaction.

Since natural plasmalogens have a high percentage of hexadecanal as the fatty aldehyde (Horrocks, 1972), we attempted to prepare cis-1-hexadecenyl bromide by the same procedures used to prepare cis-1-deceny1 bromide (XXII). Generally between 5 and 10% trans isomer was formed for this homolog, but it could not be purified by distillation. Heating the pot to temperatures in excess of 100° C (the bromide distilled at 105° C at 0.05 mm Hg) caused the distillant to polymerize and decompose, and the vinyl bromide that was collected contained up to 30% trans-isomer.

As described here, cis-alkenyl thioethers of mercapto­glycerol can be prepared by several routes. Highest yields (still only 25%) and lowest percentage of side reactions were obtained with lithium metal reduction of decenyl bromide and its subsequent reaction with symmetrical disulfide (XXI). In order to convert the alkenyl thioether (XXIV) into an ethanolamine or choline plasmalogen, the isopropylidene blocking group must be removed. Preliminary attempts with a boric acid/trimethylborate system (Mattson and Volpenhein, 1962) indicate that this step may be a problem. A product with the expected \( R_f \) of an alkylglycerol is formed, and it possesses both free hydroxyl
groups and an intact double bond (1610 cm\(^{-1}\)). However, it also exhibits a strong carbonyl stretching frequency of 1710 cm\(^{-1}\) that could be due to a hydrogen bonded ketone or an electron deficient thioester. No aldehydic C(O)H stretch was observed. Experiments have not ruled out the possibility that the carbonyl group belongs to another compound that coincidentally comigrates with the desired product on both tlc and silicic acid columns.
CHAPTER VII
SUMMARY

Sulfur substituted analogs of some glycerolipids have been chemically prepared and used to spectrophotometrically assay phospholipases A\(_1\) and C, and monoglyceride lipase. Glycerothiophosphoester analogs of phosphatidyl choline were prepared as phospholipase C substrates from mercapto-glycerol and mercaptoethanol by a four step procedure. The thiols were oxidized to disulfides, acylated, and reduced with dithiothreitol. Phosphocholine derivatives were made by classical methods for oxyphosphoesters. The phosphatidyl choline analog was converted into the phosphatidyl ethanolamine analog by transphosphatidylation with phospholipase D. The synthesized compounds were rac-1-S-phosphocholine-2,3-O-didecanoyl-1-mercaptop-2,3-propanediol (XIII\(_a\)), 1-S-phosphocholine-2-O-hexadecanoyl-1-mercaptop-2-ethanol (XIII\(_b\)), and 1-S-phosphoethanolamine-2,3-O-didecanoyl-1-mercaptop-2,3-propanediol (XIV).

Glycerothioester analogs of phosphatidyl choline and phosphatidyl ethanolamine were prepared as substrates for phospholipase A\(_1\). Triacyl mercaptoglycerol was partially hydrolyzed with *Rhizopus delemar* lipase to produce 1-S-2-O-didecanoyl-1-mercaptop-2,3-propanediol, which
was then converted by chemical methods into rac-1-S-2-0-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (XVII) and rac-1-S-2-0-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (XVIII). Thioester analogs of monopalmitin (XIX) and monodecanoin (XX) were prepared as substrates for monoglyceride lipase by selective acylation of mercaptoglycerol.

Methods were developed to synthesize optically active mercaptoglycerol from optically active isopropylidene glycerols. 1,2-Isopropylidenedeglycerol was tosylated and the tosyl group displaced with thiolacetate. Base hydrolysis and oxidation gave 1,1'-dithiobis(2,3-isopropylidene-2,3-propanediol) (XXI). (XXI) could be used as a source of mercaptoglycerol, or reacted with 1-decenyl lithium to form cis-1-S-dec-1'eny1-2,3-isopropylidene-1-mercapto-2,3-propanediol (XXIV). The latter is a stereospecific synthetic route to cis-alkenyl thioethers of protected mercaptoglycerol, and it may be useful for the preparation of a thioplasmalogen substrate for plasmalogenase.

Glycerothiophosphoester linkages of (XIIIa,b) and (XIV) were hydrolyzed by Clostridium perfringens and Bacillus cereus phospholipase C. Hydrolyses were followed spectrophotometrically with 4,4'-dithiopyridine (DTP). The reaction occurs with cleavage of the P-S bond, and, in the presence of DTP, final products are symmetrical
glyceride disulfides formed by disulfide exchange. Rates of hydrolysis of (XIIIa,b) and (XIV) were directly proportional to enzyme concentration with the release of equivalent amounts of thiol and water soluble phosphorus. DTP stimulated the hydrolysis of thiophosphoester substrates relative to didecanoyl glycerophosphocholine, but the mechanism is unknown. _C. perfringens_ phospholipase C exhibited a lag phase for the hydrolysis of (XIIIa) but not (XIIIb). Ca$^{2+}$ stimulated hydrolysis of (XIIIa) by decreasing both the lag period and the substrate $K_m$. The enzyme requires Zn$^{2+}$, and is protected against EDTA inhibition by a combination of substrate and Ca$^{2+}$. It hydrolyzes (XIIIa) 35 times faster than (XIV) and is not stereospecific. In contrast, _B. cereus_ phospholipase C exhibited neither a lag nor a Ca$^{2+}$ requirement, and has strong stereochemical specificity.

Commercial _Rhizopus delemar_ lipase contains small amounts of phospholipase A$_1$ and lesser amounts of lyso-phospholipase activities. It removes diacyl glycerophospholipids from mixtures with plasmalogens to give ethanolamine and choline plasmalogens in 94% purity. Hydrolysis of phospholipase A$_1$ (XVIII and XVII) and monoglyceride lipase (XIX) substrates by _R. delemar_ lipase was followed spectrophotometrically with DTP. The reactions occurred with cleavage of the S-C(O) bonds to produce water soluble thiols. In the presence of DTP,
final products of (XVIII) hydrolysis were mixed disulfides of lyso (XVIII) and thiopyridine. Linear enzyme dependencies were obtained over the pH range 4 to 7.5. Hydrolysis was not stimulated by DTP. Relative rates of hydrolysis of (XIX), (XVIII), and (XVII) at pH 6 were 30, 1, and 0.1 respectively. Differences in pH optima, Ca\(^{2+}\) stimulation, and heat stability for the hydrolysis of (XIX), (XVIII), and (XVII) suggest that the lipase contains several lipolytic activities, none of which are stereospecific.
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