Characterization of Mammalian Phospholipase D

A Thesis

Submitted to the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

Robert James Chalifour

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BY

ROBERT JAMES CHALIFOUR

A thesis submitted to the Faculty of Graduate Studies of
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To Lorraine

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ABSTRACT

The characteristics of phospholipase D(PLD)from rat tissue were examined. The ability of this enzyme to catalyze a phosphatidyl transfer reaction in addition to its hydrolytic activity was studied. Following this, an examination of the properties of PLD prior to detergent solubilization, was carried out. This study led to the observation that PLD is not detectable in the absence of a detergent activator. The activation of PLD by brain lipids was examined during the latter part of this study.

A phosphatidylglycerol (PG) forming activity was demonstrated in rat brain detergent extracts which required only glycerol and phosphatidylcholine (PC) as substrates. This suggested it was a transphosphatidylase reaction. The apparent K_m's for glycerol and PC were found to be 200 mM and 3.5 mM respectively. This activity was optimal at pH 6.0 and did not require divalent cations. PC was found to be the most effective phosphatidyl donor tested and the ability of detergents to activate the reaction was observed. The product of the reaction was determined to consist of a racemic mixture of 3-sn-diacylglycerophospho-1',3'-sn-glycerol when glycerolphosphate released from this lipid was analysed using the stereospecific enzyme sn-glycero-3-phosphate dehydrogenase. A direct comparison of PG formation and PLD activity in terms of inactivation by heat and parachloromercuriphenylsulfonic acid (PCMPS) and the effect of glycerol concentration provided evidence for the identity of the PG forming activity and PLD.

Phospholipase D was found to be completely undetectable in tissue fractions prior to solubilization, when assayed with a [^{3}H] phosphatidylcholine liposome substrate. The activity became apparent however after the addition of the bile salt taurodeoxycholate (TDOC). The optimal PC substrate concentration was 5 mM, the optimum TDOC concentration was 6 mM, while above these values both substances became inhibitory. A tissue survey of PLD, demonstrated its occurrence in all rat tissues. The subcellular localization of PLD was studied in brain and lung tissue of rat and it was found to be a particulate, microsomal activity. The sidedness of the enzyme was examined in rat brain microsomal membranes. By the criteria of inactivation with trypsin, pronase and PCMPS in intact microsomes, 50% of PLD was judged to be, exposed to the outer membrane surface.

The PG forming activity was utilized in order to demonstrate that PLD is also inactive towards the endogenous microsomal membrane phospholipids. Under these conditions in which the endogenous phospholipids and [^{3}H] glycerol are substrates for the formation of [^{3}H] PG, PLD was activated 10 fold by bile salts. A survey was carried out for endogenous brain lipids capable of activating PLD. The lipids which were tested at various concentrations were lysophospholipids, acidic phospholipids, gangliosides, fatty acyl CoA and oleic acid. Only oleic acid could activate PLD significantly. A variety of free fatty acids tested revealed that the unsaturated forms, oleate and palmitoleate were the most effective activators, being about 10 times more effective

than the bile salts. The optimal concentration of oleate depended directly upon the amount of microsomes present in the assay and was found to be 2 μ mol oleate/mg microsomal protein. Similarily the optimal concentration of oleate for PLD action towards exogenous PC substrate, as measured by its hydrolytic activity, was found to be dependent on the concentration of PC in the assay, being optimal at 2 μ mol oleate/ mol PC. The effect of temperature on PLD activity revealed an optimal reaction temperature of 30^{o}C, above which a dramatic drop in activity was observed. The high K_m for glycerol, 130 mM suggests that this route for PG formation would not be significant in vivo.

The lack of PLD activity in the absence of a detergent activator, may indicate that this enzyme is highly regulated in vivo. Free unsaturated fatty acids represent potential endogenous modulators of this enzyme.

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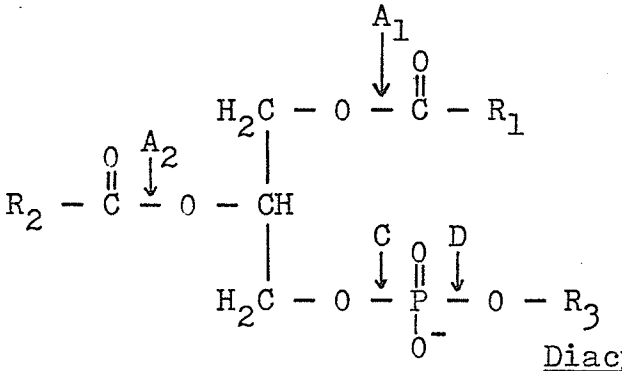
ABBREVIATIONS

$CHCl_3$	chloroform
DHAP	dihydroxyacetone phosphate
EDTA	ethylenediamenetetraacetic acid
EGTA	ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid
-GP	αglycerol phosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid
MEOH	methanol
NAD	β-nicotinamide-adenine dinucleotide
NADH	β-nicotinamide-adenine dinucleotide reduced form
NADPH	β-nicotinamide-adenine dinucleotide phosphate reduced form
PA	phosphatidic acid
PC	phosphatidylcholine
PCMPS	parachloromercuriphenylsulfonic acid
PDE	phosphodiesterase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PLD	phospholipase D
PS	phosphatidylserine
TDOC	taurodeoxycholate
TLC	thin layer chromatography
TRIS	Tris (hydroxymethyl) aminomethane

1. INTRODUCTION

The phospholipases are a group of hydrolytic enzymes which act on the phosphoglycerides. These enzymes can be anticipated to have metabolic importance given the fundamental nature of their substrates to the cell. These compounds are the main lipid components of all cellular membranes (49). In addition to this structural role, the phosphoglycerides are required for the functioning of a number of membrane associated activities (171).

The specific roles of the different types of phospholipases are far from being completely understood at this time. Four basic types of these enzymes are known to exist, which are phospholipases A_1, A_2, C and D, classified according to the bond of the phosphoglyceride molecule which they attack, as illustrated below.



R_1 - fatty acid

R_2 - fatty acid

R_3 - amino alcohol or polyol residue

Diacylglycerophosphatide Structure

The phospholipases A_1 and A_2 are carboxylic ester hydrolases, which release fatty acids from the phosphoglyceride at the carbons number 1 and 2 of the glycerol backbone. The phospholipases C and D differ from these activities in being phosphoric ester hydrolases which act on the polar head group of these lipids.

This work has been directed at the D type of phospholipase of mammalian tissues. The function of this enzyme is possibly the least understood of the phospholipases in any organism, and especially in animal tissues. It was generally accepted for some time (82), (221) that PLD did not occur in other organisms besides plants and bacteria, and as a result, very little information is available concerning this enzyme in mammals.

The potential role of this enzyme in cellular metabolism can only be speculated upon at this time. It does represent a fourth way to degrade these lipids. Its products, phosphatidic acid (PA) and choline in the case of phosphatidylcholine are rather important biological substances. PA is a compound at the major branch point in glycerolipid biosynthesis. From phosphatidic acid pathways lead to either the triacylglycerides or the zwitterionic phospholipids, phosphatidylcholine and phosphatidylethanolamine or to the acidic phospholipids, phosphatidylinositol, phosphatidylglycerol and cardiolipin. PLD potentially could have a role to play in modulating the relative content of membranes of these various lipids. PA has been found recently to have ionophoretic capabilities for the transport of calcium across phospholipid liposomal membranes (176). PA has also been found to specifically stimulate the uptake of calcium and to evoke the release of dopamine by rat brain synaptosomes (76). PA synthesis and accumulation occurred during cholinergic stimulation of smooth muscle cells in culture, and it was demonstrated that submicromolar

concentrations of this lipid could produce contractions of these cells (170). These observations have led to the suggestion that PA may mediate the release of neurotransmitters resulting from depolarization (76) and could be involved in cholinergically induced changes in membrane calcium permeability (170). Phospholipase D is also the most direct mechanism for the release of choline from phosphatidylcholine. This may be of significance in providing choline as a precursor for acetylcholine in neuronal tissues (17).

The first unequivocal demonstration of PLD in mammalian tissues was made using a detergent extract from rat brain membranes (169). Certain properties of this enzyme were described and more recently it was further characterized after partial purification from these extracts (195). No information however, is yet available concerning the properties of this enzyme prior to its solubilization from rat brain. PLD is unusual in this regard, since enzymes generally are studied preliminarily in crude states before being purified. The properties of PLD reported here provide some explanation for why this was not done for PLD.

Previous attempts to examine PLD of animal tissues were made, but no characteristics of the enzyme have been reported. Dils and Hubscher observed that liver homogenates incubated with calcium produced increased quantities of PA (45). At the time of these studies thin layer chromatographic methods were not available for the identification of phospholipids. These workers used the alkaline deacylation product, α-glycerol phosphate to identify PA. In more recent (196)

experiments using mouse liver prelabeled with [^{32}P], Taki and Matsumoto reported a calcium dependent increase in PA after incubation at 37°C. Neither of these reports were followed up beyond this initial suggestive evidence for the presence of PLD.

The major aim of this study has been to carry out investigations of the properties of PLD as it is originally found in tissue homogenates. The specific goals of this work and why these studies are important to understanding this enzyme are as follows.

1. The properties of membrane enzymes are known in some cases to be altered by detergent extraction procedures. Differences have been noted in terms of pH optima, K_m's, substrate specificity and stability of certain activities (27). It is possible therefore that the properties of PLD described in the initial tissue homogenate may more accurately reflect its properties <u>in vivo</u>.

2. There is no information available concerning the subcellular or tissue location of this enzyme. PLD of plants is primarily a soluble activity (155) while other phospholipases of both the A (202) and C (55) types in animals exist in both particulate and soluble forms. Subcellular and tissue surveys were therefore carried out.

3. During these studies, a striking requirement for detergent by PLD was demonstrated. This may explain why the enzyme was not previously studied in more detail. The reasons for this requirement, and the possible existence of endogenous lipids capable of activating PLD were

investigated. This property may suggest that PLD is a highly regulated enzyme activity.

4. During the initial parts of this work, PLD present in detergent extracts of brain membranes was found capable of catalysing a polar head group exchange reaction, in addition to its hydrolytic activity. Although probably of little physiological significance this exchange reaction provided a valuable second assay for PLD.

LITERATURE REVIEW

This section presents an overview of current knowledge concerning phospholipase D (PLD). Aspects of mammalian phosphoglyceride metabolism are discussed as a basis for understanding the role PLD may play in the cell. A number of specific questions regarding PLD of rat tissues are investigated in this study. One concerns whether PLD from this source can catalyse a polar head group exchange reaction in addition to its hydrolytic activity. Another point studied was the role that detergents play as activators of this enzyme *in vitro*. Part of this literature review has therefore been devoted to presenting background literature relevant to these questions.

Significance of the Phosphoglycerides

The phosphoglycerides are important primarily because of their role in biological membrane structure and function. Phosphoglycerides are the predominant membrane lipids (49) and form the basic framework of membranes, the lipid bilayer. A current concept of membrane structure, the Fluid Mosaic Model (180) visualizes membranes as having proteins in or on this bilayer as the main functional components, surrounded by lipid regions serving to maintain the integrity of the structure. In addition to this role of forming the bulk phase of the membrane, other roles in the interaction with membrane proteins exist. Phospholipids may modulate membrane enzymes and other activities through these interactions. This concept is illustrated by examples such as the integral

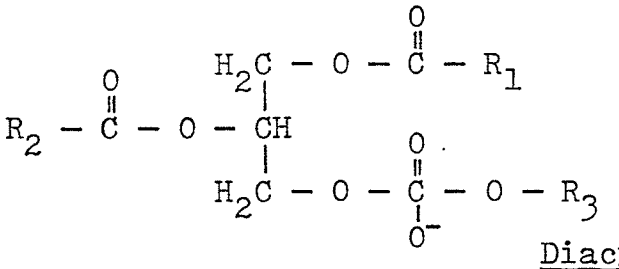
mitochondrial membrane enzyme β-hydroxybutyrate dehydrogenase (BDH). This enzyme has an absolute requirement for phosphatidylcholine and studies have shown that one of the parameters affected by lipid binding is the interaction of BDH with its cofactor. BDH shows little binding of NADH except in the presence of the phospholipid (64).

Certain phospholipids have been implicated in mechanisms of signal transmission across membranes. Methylation of phosphatidylethanolamine has been demonstrated in response to a number of cell surface events such as binding of catecholamine neurotransmitters and lectins (85). This methylation is postulated to be coupled to calcium influx and release of arachidonic acid for subsequent prostaglandin synthesis in many cell types. It is postulated as a common initial pathway for transduction of many receptor mediated biological signals through membranes (83). The involvement of inositol phospholipids has long been speculated in the functioning of certain neurotransmitters with which calcium influx into cells is coupled (129). Recent studies have demonstrated a calcium ionophoretic capability of phosphatidic acid with liposomes as model membranes (176). Such a role for this phospholipid may be of great significance in the action of hormones and the functioning of the central nervous system. In rat brain, phosphatidate, but not other phospholipids can stimulate calcium uptake and cause an associated release of the neurotransmitter dopamine from synaptosomes (76). The cholinergic stimulation of cultured smooth muscle cells caused a rise in their content of phosphatidic acid (170).

Low concentrations of this lipid could cause contraction of these cells, thus implicating phosphatidic acid in the mechanism of cholinergic receptor linked ion transport (170).

In lung, phosphatidylcholine and phosphatidylglycerol are major components of the surfactant material which is necessary for stabilization of the alveoli (162). In liver tissue phosphatidylcholine is synthesized as a component of bile where it functions as an emulsifier (88). Also in liver phosphatidylcholine is produced for use as a structural component of lysoproteins which are secreted by this organ (88).

The phosphoglycerides occur in many structurally diverse forms in terms of fatty acid and polar head group composition. These compounds share the generalized structure shown here (127).



R_1 - fatty acid

R_2 - fatty acid

R_3 - amino alcohol or polyol residue

Diacylglycerophosphatide Structure

In addition the ethanolamine phosphoglycerides and to a lesser extent other phosphoglycerides contain along with this diacyl ester structure, 1-alkyl-2-acyl-sn-glycerol-3-phosphoryl and 1-alkyl- 1′-enyl -2-acyl-sn-glycerol-3-phosphoryl (plasmalogens) analogues (185).

Synthesis of Phospholipids by Polar Head Group Exchange

The biosynthesis of the phosphoglycerides has been reviewed quite recently (10) and also on earlier occasions (116,201,186). The interconversion of phospholipids by exchange of polar head groups represents a mechanism for the formation of one type of phospholipid at the expense of another. In other words, no net synthesis of phospholipids takes place. The relationship of these activities to PLD has been questioned since in both cases the same bond of the phosphoglyceride is involved. These reactions were described early in the study of phospholipid metabolism. Exchanges incorporating choline (44), ethanolamine (18), serine (90), and inositol (143) have been described in mammalian tissues.

Inositol exchange was originally noted in rat liver (143) and since has been reported in pig thyroid (100), rat brain (99), rat lens tissue (21) and rat lung tissue (15). CDP-diglyceride inositol transferase requires either magnesium or manganese ion for activity while the inositol exchange is active in the presence of manganese ion only. Further differences between these two activities in rat liver such as in pH optima, in sensitivity to inhibitors and in dependence on exogenous lipids have been reported, indicating that the inositol exchange is catalysed by a specific enzyme (193,15). This enzyme was recently purified (192) and evidence has been presented indicating that it actually represents an exchange of the myoinositol moiety of phosphatidylinositol for free inositol, and therefore results in no net synthesis (192).

The other exchange activities are similar to inositol

exchange in being non energy requiring, but differ in requiring calcium ion rather then manganese ion for activity. These activities are also widely distributed, having been described in many mammalian and other tissues (102). Workers in this field have for many years tried to determine whether choline, ethanolamine and serine exchanges are catalysed by specific enzymes. Another point of controversy has been whether these reactions are catalysed by phospholipase D in an exchange rather then hydrolytic reaction.

The probable identity of ethanolamine and serine exchange activities of chick brain was suggested (149). This conclusion was based upon similar inhibitory effects of heavy metal ions, similar pH optima, and competition between ethanolamine and serine during their respective reactions. Different properties were however observed for the three exchange activities when studied in rat brain tissues (101). Differences were observed in pH optima, stability to storage at 4°C, phospholipase A, C and D treatment of the microsomes, and inhibition by a series of structural analogues. Clear evidence for the existence of separate exchange enzymes was obtained by the chromatographic separation of distinct enzyme fractions for each of the three alcohols, choline, ethanolamine and serine after their solubilization from rat brain membranes with detergents (166, 168,132). An L-serine specific base exchange enzyme was purified 37 fold from rat brain microsomes (194,197). It was found devoid of both choline and ethanolamine exchange activities and in addition did not contain detectable phospholipase D activity. This enzyme greatly preferred ethanolamine

phospholipids over other types tested.

The physiological significance of these reactions is not known at this time. The serine exchange activity represents an important route for phosphatidylserine synthesis in animal tissues (227,41) since the de novo pathway described in bacteria for this phosphoglyceride (103) involving a CDP-diglyceride : L-serine phosphatidyltransferase has not been demonstrated in animal tissues (41). It should be noted that recently a second, energy dependent reaction producing phosphatidylserine has been described in rat brain tissue (154). This may be similar to the energy dependent biosynthesis of this lipid observed by Hübscher et al. in rat liver (90). The mechanism of this energy requiring reaction is not known. Experimental results from studies seeking to determine the extent of these exchanges in vivo have provided inconclusive results. A number of workers concluded that the reactions do contribute to phospholipid metabolism (199,191,142), while others believe that the reactions are not significant (3 ,12). Relatively small pools of the membrane phospholipids, 0.5-9% have been estimated to be substrates for these exchange activities (58,150). It seems reasonable to postulate that these enzymes will be found to have more subtle roles in phospholipid metabolism, rather than simply the production of certain phospholipids. In light of the numerous membrane activities requiring phospholipids for activity (119), these base exchange enzymes have potential as regulatory mechanisms, acting by altering the character of the phospholipid head group. Studies to test this type of function of the base

exchange enzymes have provided indications that the uptake of γ-aminobutyric acid by synaptosomes is stimulated by serine exchange (40). Examination of another activity, adenylate cyclase of brain synaptosomes indicated that ethanolamine exchange produces a decrease in the NaF-stimulated production of cAMP (54).

The Degradation of Phospholipids

Mammals are known to contain a variety of phospholipase activities, consisting of phospholipases A_1, A_2, B, C and D as well as lysophospholipases A and D. In addition, the reversal of certain biosynthetic enzymes such as CDP-diacylglycerol:inositol transferase and choline and ethanolamine phosphotransferases may be significant degradative pathways under certain conditions.

At least two general pathways for the complete degradation of phospholipids exist (198). One consists of the initial hydrolysis of the fatty acid moieties, which results in the production of a glycerol phosphoryl alcohol phosphodiester compound. This product is further degraded by the action of a phosphodiesterase (213) and phosphatase. An alternate route consists of the initial hydrolysis of the phosphate moiety, which results in the production of diglyceride. Diglyceride would then be degraded by the action of two enzymes, diglyceride lipase and monoglyceride lipase (23).

Phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2) have been found in a wide range of mammalian tissues and also in several subcellular locations (202). These activities

have been most intensively studied in liver where it was found that the properties of the enzyme vary with their locations, indicating the existence of isozymes. Plasma membrane have both PLA_1 and PLA_2 activities which are calcium dependent, while lysosomes have both, but neither requires calcium. The cytosol has both of these activities, however, the divalent cation requirements are controversial in that case. Microsomes only have a calcium dependent PLA_1 activity, while mitochondria only have a calcium dependent PLA_2 activity (202).

Phospholipase A_1 has been purified from a number of mammalian sources (203,217). It has been noted with a purified pancreatic form of the enzyme that this enzyme exhibited phospholipase B activity (203) which consists of both phospholipase A and lysophospholipase activities (127). This seems to be a general property of the mammalian and bacterial PLA_1 enzymes. Interestingly, it was noted that the conditions of assay greatly influenced the substrate specificity of this enzyme. When the homogeneously pure pancreatic PLA_1 was assayed in the presence of high concentrations of deoxycholate, it was apparently a PLA_1 type of enzyme. However, with intermediate amounts of this detergent, the enzyme displayed both activities and therefore acted as a phospholipase B(203).

PLA_2 has been purified from a range of mammalian tissues including rat spleen, (156) human pulmonary secretions (165), platelets (5) and sheep erythrocytes (113). This enzyme differs from the PLA_1 type in being very specific for the

sn-2 position of phosphoglycerides.

A number of functions are attributed to the phospholipase A's including the liberation of precursor fatty acids (117) for prostaglandin biosynthesis, digestion of dietary lipids (88), taking part in the synthesis of some molecular species of phospholipids by deacylation-reacylation pathways (88) and the turnover of membrane phospholipids (38). The regulation of the phospholipase A's is not understood in great detail at this time. The evidence available indicates that a number of mechanisms are involved including covalent modification, and interaction with regulatory proteins. Pancreatic PLA_2 occurs as an inactive zymogen which is activated by proteolytic cleavage of a heptapeptide from the N-terminus (39). Quite recently a PLA_2 inhibitor protein has been discovered (13) and purified (84). This protein now called lipomodulin, was found to be induced by anti-inflammatory steroids (51) and it is believed that by inhibiting fatty acid release it prevents prostaglandin synthesis, explaining the mechanism of the action of these steroids. A number of activator proteins have been described for PLA. Preliminary evidence has been obtained indicating that the calcium binding protein calmodulin activates the platelet PLA_2 (220). Apolipoprotein C-11 stimulates the PLA_1 activity of lipoprotein lipase (71) while lecithin-cholesterol acyltransferase which can exhibit PLA_2 activity when cholesterol is absent, is completely dependent on apolipoprotein A_1 (48).

Phospholipase C can be classified into two main types in mammalian tissues. The first type is a phospholipase C specific for

phosphatidylinositol (PLC-PI) which has been known for many years (182). The other type only recently observed to occur in mammals, (126) is a phospholipase C with a broad substrate specificity towards phospholipids (PLC-G). Both soluble and particulate forms of PLC-PI occur in animal tissues and are found to require calcium ion for activity (93,120). Lysosomes also contain a second form of PLC-PI which is not dependent on calcium ion for activity (95). A phospholipase C capable of hydrolysing phosphatidylethanolamine reported in rat brain (216) did not require divalent cations and was located in the microsomal fraction. A lysosomal PLC-G was very recently described (126) which did not require cations either and was demonstrated to occur in a wide range of rat tissues (89). It is possible that the lysosomal PLC-PI and PLC-G are in fact the same enzyme. Lysosomal PLC-PI could hydrolyse phosphatidylcholine and ethanolamine at only 5 and 1.5% respectively, of the rate it hydrolysed phosphatidylinositol (95). The lysosomal PLC-G acted on phosphatidylcholine and ethanolamine at 31 and 10% respectively of the rate it acted on phosphatidylinositol (126).

The physiological roles of PLC-G are not known. This activity represents a route of phospholipid breakdown which avoids the production of lysophospholipids, compounds which can effect a wide range of physiological processes (215) and which can be cytotoxic (215). PLC-G, since it is a lysosomal activity (126) may be involved in the degradation of lipoprotein derived phospholipid entering these organelles after

endocytosis (66).

The PLC-PI has been widely implicated in the well known phenomenon of physiologically stimulated phosphatidylinositol turnover in animal tissues (129, 78), during which various stimulations bring about an increased incorporation of labeled phosphate into phosphatidic acid and phosphatidyl-inositol. Interactions of many receptors with their ligands precede this effect (129). Most, but not all, workers agree with the suggestion (87) that one of the initial steps in this phenomenon is the breakdown of phosphatidylinositol to diglyceride, catalysed by PLC-PI. The resulting diglyceride can be phosphorylated to phosphatidic acid, a step which is believed to result in the high incorporation of radioactive PO_4 into this lipid. PA can then be converted to phosphatidyl-inositol, with retention of the phosphate label, explaining the high labeling of that lipid. The physiological significance of this effect is not clearly understood, however a widely studied hypothesis proposed by Michell (130) suggests it is involved in the mechanism of calcium gating through biological membranes.

The observation that the non lysosomal PLC-PI is relatively inactive toward a membrane-bound substrate (128, 92) suggests that the physical-chemical state of the membrane phospholipids or their accessibility on the membrane may be a controlling factor of this activity. The finding that free unsaturated fatty acids (96) and phosphatidic acid (97) greatly stimulate this enzyme towards the membrane substrates, has led to the suggestion that these substances may regulate PLC-PI in vivo

(94).

The reversal of certain biosynthetic enzymes for phosphatidylcholine, ethanolamine and inositol may provide another degradative route for these phospholipids. The reversibility of CDP-choline:diacylglycerol choline phosphotransferase and CDP-ethanolamine:diacylglycerol ethanolamine phosphotransferase has been demonstrated in rat brain, (67,68) rat lung (174) and rat liver (104). In the presence of CMP the microsomal enzymes are capable of the following reaction.

PC (PE) + CMP $\rightleftharpoons$ CDP-Choline (CDP-ethanolamine) + Diglyceride

The diglyceride produced can be subsequently degraded by the action of diglyceride and monoglyceride lipases (23). The quantitative importance of this reaction in vivo has yet to be evaluated.

Reversal of CDP-diglyceride:inositol phosphatidyltransferase has been demonstrated (145). In the presence of CMP the enzyme catalyses the reaction:

PI + CMP $\rightleftharpoons$ CDP-diglyceride + myo-inositol

The further breakdown of CDP diglyceride can take place via another enzyme, CDP-diglyceride hydrolase, recently demonstrated to be present in mammals using brain lysosomes (160). The reaction catalysed by this enzyme is:

CDP-diglyceride + H_2O $\rightleftharpoons$ PA + CMP

The phosphatidic acid (PA) can be degraded further by either phosphatidic acid phosphatase or by a phospholipase A activity (11).

Phospholipase D

For many years phospholipase D (PLD) was believed to be an enzyme present exclusively in the higher plants (221). Today, however, it has been established to occur in most types of organisms (82). The activity was first described over 3 decades ago in extracts of carrot root and cabbage leaves (74). A broad distribution of the enzyme in many plant species has been subsequently demonstrated (155,205,47). Prokaryotic cell types were shown to have the enzyme also when it was detected in Corynebacterium pyogenes in 1963 (53). Simple eukaryotes also have this enzyme as shown by studies reporting its presence in red algae (4), baker's yeast (70), slime mold (82) and fungi (225). Early attempts to document the occurrence of PLD in mammalian tissues using rat liver (45) and mouse liver (196) were not conclusive.

The initial demonstration of PLD in animal tissues was made in 1975 (169) when the activity was observed in a detergent extract of rat brain membranes. Since then PLD has been reported in human (105) and rat (118) eosinophilic leukocytes and it has been partially purified from rat brain (195) and human eosinophils (105). Mammalian tissues were also demonstrated to contain a lysophospholipase D (Lyso PLD) (224). The properties of this activity clearly distinguish it from the PLD acting on diacylglycerol phospholipids.

Properties of Mammalian PLD and Lyso PLD

The properties of mammalian PLD were originally examined in a detergent extract obtained from a rat brain

particulate fraction (169) and more recently have been re-examined after partial purification from this extract (195). Due to the previous inability to detect the activity of this enzyme in tissue homogenates, unless extracted with detergents, phospholipase D has not been characterized in a membrane bound form. The work reported in this thesis represents the first study of the properties of PLD under these latter conditions, which may more closely reflect the in situ characteristics of this enzyme.

In the initial detergent extracts, the enzyme was assayed by measuring the conversion of [U-^{14}C] phosphatidylcholine to [^{14}C] phosphatidic acid, a specific assay for PLD. The measurement of choline release is an unreliable assay for PLD in impure preparations since the action of other degradative enzymes in multi-step reactions can also release choline. This could occur via the sequence PLA, lysophospholipase A and phosphodiesterase. In these extracts PLD activity is optimal at pH 6.0, and does not require divalent cations as judged by the lack of inhibition of its activity by EDTA at a 12 mM concentration. 5 mM Calcium or magnesium chloride activate the activity by 50%. The apparent K_m for phosphatidylcholine is 0.8 mM with a V_{max} of 10 nmol·mg^{-1}·hr^{-1}. The enzyme cannot release choline from p-nitrophenylphosphorylcholine, and so it may have a requirement for a lipid group in its substrate. The presence of an essential sulfhydryl group is suggested by the sensitivity of PLD to the sulfhydryl reagent parachloromercuriphenylsulfonic acid (PCMPS), which at a 60 μM concentration nearly completely inhibited the activity

(169).

The solubilized PLD activity of rat brain has been partially purified. The activity was enriched 240 fold from the initial extract using ammonium sulfate precipitation, Sepharose 4B gel filtration and two sequential DEAE cellulose column chromatographic steps. The final fraction had a specific activity of 120 $nmol \cdot mg^{-1} \cdot hr^{-1}$ and was found to have characteristics similar to those of the enzyme in the initial extract. The pH optimum was 6.0, EDTA inhibited the activity only slightly and calcium ion stimulated by about 65%. The enzyme showed a slight preference for choline phospholipids, K_m phosphatidylcholine, 0.75 mM; K_m dipalmitoyl phosphatidylcholine, 0.78 mM; K_m phosphatidylethanolamine, 0.91 mM. An approximate molecular weight of 200,000 was estimated by gel filtration of the activity (195).

The presence of PLD in human eosinophil polymorphonucleocytes was reported (105). There is a problem in interpreting this result because a non specific assay procedure was used throughout this work, which was based upon the release of free choline. The activity of this PLD was higher by a factor of 100 in the initial eosinophil homogenate compared to the initial detergent extract of rat brain, $1 \mu mol \cdot mg^{-1} \cdot hr^{-1}$ compared to 10 $nmol \cdot mg^{-1} \cdot hr^{-1}$, respectively. The eosinophil PLD in an apparently soluble form was purified by a factor of 162 using DEAE anion exchange, Sephadex G100 gel filtration, CM cation exchange and isoelectric focusing. A molecular weight of 50,000 was estimated by gel filtration and the activity was optimal at pH 4.5-6.0. Further characteristics

of this enzyme were not reported.

A lyso PLD was detected in rat brain tissue initially (224) and since then has been found to be present in other rat tissues with highest activity in liver and testis (222). The properties of this enzyme clearly distinguish it from the diacylphosphoglyceride PLD also present in rat tissue (169). Unlike that enzyme lyso PLD is detectable in intact tissue fractions but becomes undetectable after detergent solubilization (224), lyso PLD requires Mg^{2+} for activity and is inhibited by calcium ions. The optimum pH was 7.0-7.6 and in contrast to PLD it was relatively insensitive to PCMPS, being inhibited only 43% by a 1 mM concentration of the sulfhydryl reagent. Subcellular fractionation of liver lyso PLD indicated that this enzyme is located in the microsomal fraction (222). The substrate specificity of this enzyme were recently reported (223). Lyso PLD was found to be specific for ether linked lysophosphoglycerides. 1-Alkyl-sn-glycero-3-phosphocholine or ethanolamine were hydrolysed at about the same rate while the corresponding 1-acyl linked lyso analogs and the 1-alkyl-2-acyl analogs were not hydrolysed by the enzyme (223).

Properties of Phospholipase D from Non Mammalian Sources

PLD has been purified to homogeneity from plant sources, peanut seed (80), cabbage (1), and from microbial sources, Streptomyces chromofuscus (91), Streptomyces hachijaensis (140) and Bacillus subtilis (61). A number of partial purifications have also been reported for the enzyme from a range of

sources (82). The success of these purifications is due in part to the fact that these are all soluble forms of the enzyme. Particulate forms found in beets, spinach or cabbage plastids (110,107) have not been purified. A particulate form from red algae (4) was resistant to solubilization and purification. The molecular weights reported from these purifications show a great deal of variation in size of PLD. Cabbage 112,500 ± 7500; peanut seed, 200,00 ± 10,000; Streptomyces chromofuscus, 50,000; Streptomyces hachijaensis, 16,000; Bacillus subtilis, 21,500 ± 300.

In plants an acidic pH optimum is generally noted which ranges from pH 4.8-6.0 (82). The microbial forms of the enzyme on the other hand are optimal at slightly alkaline pH values, pH 7.0-8.5 (140,61). The optimum pH of the plant enzyme was found to vary as a function of substrate properties. When phosphatidylcholine was dispersed by ultrasound, a pH optimum of 4.9 was seen. If ether was used to disperse the phosphatidylcholine, the optimum was 5.2, while if detergents were used to disperse the substrate the optimum was pH 6.5 (35).

PhospholipasesD from plant sources have a requirement for calcium ions for activity. Early reports that the particulate form of the enzyme from plastids was calcium independent, (106,107) were explained by the presence of sufficient endogenous calcium (110,60). Other cations can substitute for calcium in some cases. The soluble cabbage enzyme was also activated by strontium, barium and zinc

cations to 67, 34 and 12% of the activity observed with calcium (31). The cotton seed enzyme similarly was activated by a range of cations however the order of effectiveness varied slightly: strontium > calcium > barium > manganese > zinc (158). Phospholipase D from red algae, a particulate enzyme, did not require cations although it could be slightly stimulated by the presence of calcium, strontium or magnesium ions (4).

The cation requirements for the microbial enzymes studied are similar. PLD from Corynebacterium ovis did not require cations for activity (188) while the enzymes from other microbes did require cations, and, as with plants, various types of cations could fill the requirement (141,26).

Phospholipase D from plant sources is found to have a wide specificity of action towards phospholipids. In contrast, the microbial enzyme, in certain cases, has been found to be quite specific for one or two lipids. Studies with the cabbage enzyme revealed it capable of hydrolysing many diacylphospholipids (108), and in addition monoacylglycero-phosphorylcholine (lysolipid), 1-alkenyl-2-acyl glycero-phospholipid (plasmalogens), dialkyl derivatives and even sphingomyelin (31,32). The rate of hydrolysis of the 1-alkyl-2-acyl and 1-alkenyl-2-acyl-sn-glycero-3-phosphoryl-choline was found to occur at rates one tenth to one third that observed for the diacyl phosphatidylcholines (206). It has been generally believed that in spite of this wide specificity, phosphatidylinositol was not a substrate for PLD. This was recently re-examined and found incorrect (25). Phosphatidyl-

inositol is readily attacked by cauliflower PLD. The previous inability of others to detect this reaction (60,82) is explained by the fact that while some phosphatidic acid is formed, the main products are bis (phosphatidyl) inositol, most likely produced by a transphosphatidylation reaction between two substrate phosphatidylinositols (25).

The substrate specificity of microbial PLD is dependent on the species from which it is obtained. PLD from Streptomyces chromofuscus is an enzyme with broad substrate specificity.It was able to hydrolyse lecithin, lysolecithin, sphingomyelin and phosphatidylethanolamine. The relative rates of hydrolysis were found to be lysolecithin, 100%; lecithin, 87%; and sphingomyelin 22% (91). The PLD from Hemophilus parainfluenza is an example of a specific PLD. This enzyme will hydrolyse cardiolipin but not phosphatidyl-ethanolamine, glycerol, serine or choline (141). The enzyme from Corynebacterium ovis is another example of a specific PLD. This enzyme acts on sphingomyelin and lysolecithin but not on any other glycerophospholipid whether in pure or protein bound form (188).

Phospholipase D Catalysed Polar Head Group Exchange

This section discusses evidence that PLD can catalyse a transphosphatidylation or polar head group exchange reaction. Whether PLD catalysed exchange is the same as the exchanges for choline, ethanolamine or serine, known as base exchange, has been controversial (82). At this time evidence indicates that those two types of exchanges are catalysed by distinct

proteins.

These exchange reactions, as previously discussed, represent a mechanism for the interconversion of phospholipids by substituting one polar head group alcohol for another. This reaction can be written as:

$$\text{phosphatidyl-}R_1 + R_2\text{-OH} \rightleftharpoons \text{phosphatidyl-}R_2 + R_1\text{-OH}$$

The question of the role of PLD in such reactions is a long standing one proposed originally by Hubscher and co-workers (44). They first reported head group exchange reactions in rat liver and based their suggestion for an involvement of PLD on the knowledge that most hydrolytic enzymes such as the proteases (56), the phosphatases (133) and the glycosidases (208) can catalyse the transfer of alcohols in addition to their more usual hydrolytic reactions. An alternative to this suggestion is that there exist specific proteins which catalyse the transfer of head group alcohols. If the observed exchanges are due to specific enzymes then they may have definite functions in the cell. On the other hand if the activities are both catalysed by PLD it would be necessary to ask whether this enzyme catalyses primarily a hydrolytic reaction and under what situations it catalyses a transfer reaction.

The strongest data on this point has come from purifying the enzymes in question and determining whether exchange and hydrolysis activities co-purify. Studies of this sort have been carried out in both plant and animal systems.

Evidence for Non-Identity of PLD and Base Exchange Enzymes in Animal Tissues

Dils and Hubscher in 1959 (44) were the first to note an energy independent, calcium requiring phospholipid head group exchange reaction, during which labeled choline was incorporated into rat liver phospholipids. These investigators suggested the possibility of PLD catalysing the reaction (44) but were unable to find strong evidence for this type of hydrolytic activity in animal tissues (45). The likelihood of PLD being involved therefore seemed small. In 1975 however, with the successful demonstration of PLD in rat brain (169), the Dils and Hubscher suggestion became a distinct possibility. The properties of PLD and the base exchange enzymes, however, were not found to be the same when compared thus suggesting that different enzymes were responsible. PLD has an acidic pH optima and does not require calcium ions for activity (169), whereas in contrast, the base exchange activities have alkaline pH optima and a definite calcium ion requirement (101).

More conclusive evidence for the non-identity of these activities was obtained by the actual separation of the proteins catalysing each reaction. An L-serine-phospholipid base exchange enzyme was solubilized from rat brain microsomes with detergents and purified using Sepharose 4B gel filtration and DEAE ion exchange chromatography (194). The final active fraction, 37 fold enriched, was free of ethanolamine and choline exchange activities. Using phosphatidylcholine and phosphatidylethanolamine as substrates, no PLD activity could

be detected in the serine exchange enzyme fraction (194).

Following this, a complementary approach was taken to provide further evidence in support of this observation. Rat brain phospholipase D was purified by a separate procedure to a stage which was free of any of the three base exchange activities (195), again demonstrating that different proteins are involved in these two reactions.

Evidence for the Non-Identity of PLD and Base Exchange Enzymes of Plant Tissue

The studies which have been carried out with plant tissues, in agreement with the results obtained from the studies of rat brain tissue, indicate that PLD and base exchange activities are catalysed by separate enzymes. Studies have revealed that plant PLD can catalyse a phospholipid polar head group exchange reaction, which is however, distinctly different from the base exchange enzyme since it is non-specific and displays very low affinity for the alcohols being exchanged.

Evidence that PLD can catalyse an exchange reaction was initially presented in 1967 (226). Yang and co-workers purified cabbage PLD 110 fold and found that an ethanolamine exchange reaction co-purified. This exchange appeared different from base exchange in having a very high K_m for ethanolamine. This exchange reaction shared the same pH dependence, calcium requirements, and similar inactivations while stored at $4^{o}C$ and when treated with the inhibitor, PCMPS. The PLD catalysed exchanged occurred with many

alcohols and was therefore rather non-specific. The enzyme displayed a low affinity for the alcohols in each case. The amount of alcohol which resulted in 50% maximal exchange reaction was 147 mM for ethanol, 50 mM for ethanolamine, 120 mM for glycerol and 950 mM for serine (226).

The reaction mechanism of this exchange was investigated using an isotope exchange experiment with $[^{14}C]$ choline (226). Labeling of phosphatidylcholine occurred in the presence of phosphatidylcholine and enzyme, but not in the presence of phosphatidic acid and enzyme. This demonstrated that the reaction consists of a transesterification between an esterified alcohol at the phosphate group and a non-esterified alcohol in solution. The reversal of PLD is ruled out since phosphatidic acid failed to participate in the isotope exchange reaction. Considering the proposed mechanism for other hydrolytic enzymes (57), and the evidence that the two reactions are catalysed by the same enzyme, Yang et al. (226) proposed the following reaction mechanism for PLD.

1. Phosphatidylcholine + enzyme $\rightleftharpoons$ phosphatidyl-enzyme + choline

2a. Phosphatidyl-enzyme + $H_2O \rightleftharpoons$ phosphatidic acid + enzyme

2b. Phosphatidyl-enzyme + alcohol $\rightleftharpoons$ phosphatidylalcohol + enzyme

The ability of PLD from plants to catalyse polar head group exchange has been confirmed by a number of other laboratories. Dawson in 1967 prepared a highly purified cabbage PLD and demonstrated the ability of the enzyme to transfer various aliphatic alcohols such as glycerol,

ethanolamine, methanol and ethylene glycol to the phosphatidyl unit of phosphatidylcholine (34).

Tzur and Shapiro purified PLD from peanut seed 1000 fold and demonstrated that a polar head group exchange activity, measured with methanol as substrate, co-purified throughout the procedure (200). Quite recently Rakhimov et al. (157) prepared a highly purified PLD from radish and demonstrated its ability to catalyse a transfer reaction with methanol. The specificity of cabbage PLD for the alcohol group has been systematically investigated (112) demonstrating that a wide variety of alcohols can be transfered.

Plants are known to also contain exchange activity which appear distinct from the reaction catalysed by PLD. This activity was first demonstrated in plants in 1966 (131) and is found to be similar to the base exchange activity of animals. Ethanolamine, serine or choline were incorporated into pea seedling phospholipids (204). The activity required calcium ion at 3 mM concentration and was optimal at pH 8.5. The exchange reaction showed high affinity for ethanolamine as substrate $K_m = 4 \mu M$.

A direct comparison of plant PLD and plant base exchange activity was made with a cabbage preparation (167). In this system ethanolamine and choline exchange, $K_m =$ 1.25 mM and 2.5 mM respectively had a pH optima of 9.0, while the hydrolytic activity of PLD was optimal at pH 6.0. At pH 6.0 no exchange could be detected in the presence of the low ethanolamine and choline concentrations used (167). Some further differences documented between PLD and base exchange in this cabbage

preparation were in calcium ion requirements. Base exchange activity was optimal at 4 mM calcium while PLD required a much higher level, 28 mM. Base exchange activities were found to be inhibited by hemicholinium-3 a choline analogue, K_i = 1.25 mM for ethanolamine exchange and K_i = 2.5 mM for choline exchange. PLD was not sensitive to this compound. Differences were also demonstrated in the heat stabilities of PLD and base exchange (167).

In summary the evidence indicates that the base exchange activities with high affinity for choline, ethanolamine and serine exist in both plant and animal tissues. In both tissues PLD has been shown to have different properties from these exchange activities. In plants however, PLD does catalyse a similar exchange of head groups, but this activity is distinct from the base exchanges for choline, ethanolamine and serine. PLD catalyses a low affinity, non-specific exchange, while the other exchanges are high affinity and specific for certain alcohols. The glycerol exchange activity reported in this thesis appears to be the first demonstrated exchange reaction catalysed by mammalian PLD.

Detergent Activation of Enzyme Activities

At the beginning of this study it was noted that one of the most striking features of mammalian PLD was that, while its activity is undetectable in intact tissue fractions, it can be detected in detergent extracts of these tissue fractions (169). This may explain the reported absence of PLD in mammalian tissues (221) long after it was found in most

other organisms. Controlling the activity of hydrolytic enzymes such as PLD is necessary because of its potential to breakdown cellular membranes. It seems possible that the lack of PLD activity in non-solubilized tissue fractions may reflect regulatory mechanisms for this enzyme.

Detergents activate a number of other enzymes. The mechanism of these activations is not in all cases the same.

When the substrate binding site of an enzyme is located within a closed vesicle, impermeable to substrate molecules, then little or no activity will be detected during an assay. Substances which disrupt the membrane barrier, such as detergents, will under these circumstances be observed to cause an enzyme activation.

Lysosomal enzymes are a classic example of this phenomenon. The many hydrolytic activities contained in these organelles become detectable when the lysosomal membrane is disrupted by detergents and other treatments (211). Mitochondrial ATPase stimulation by high free fatty acid concentrations has been known for many years (153) and is believed to result from detergent effects on the mitochondrial membrane (218). There are a number of cases of this form of detergent activation of microsomal enzymes. This is due to the fact that these structures are closed vesicles with limited permeability (139), especially to large or charged molecules.

One of the best understood examples of such a detergent stimulated microsomal enzyme is glucose-6-phosphatase. When this enzyme is assayed with the impermeable substrate, mannose-6-phosphate, it displays very low activity. A 10-16

fold activation of this enzyme is observed when detergents are added to its assay (6). There is evidence that the detergents act to allow the substrate access to the enzyme. This activation was found to correspond to increased membrane permeability as monitored by EDTA penetration (6). Furthermore, glucose-6-phosphatase has been localized on the lumenal or inner surface of microsomal vesicles by proteolysis studies (138) and also by product localization (121). Mannose-6-phosphatase latency in the absence of detergents is now widely used as an index of microsomal membrane integrity (9,6). There are a number of other examples of microsomal enzymes that are activated because they are located within the microsomal vesicle, separated from their substrate. These are ethanol acyltransferase of rat liver (147), alkyldihydroxyacetone phosphate synthase of harderian gland and ascite carcinoma cells (161), nucleoside diphosphatase of rat liver (115), and β-glucuronidase of rat liver (42). 5^1-Nucleotidase is an example of an enzyme which is activated by detergents as a result of being located within a secretion vacuole (123).

The detergent stimulation of enzymes acting on lipid substrates has been widely noted (63). These substrates often belong to the group of lipids classified as insoluble swelling amphiphilic lipids (183), which includes the phosphoglycerides. When placed in an aqueous environment, these compounds form large multibilayered liposomes. In order for these structures to be effectively acted on by enzymes, they often must be further dispersed or solubilized,

usually by a detergent (36). Examples of enzymes activated by detergents in this way are numerous, including phospholipases A (59), C (97) and D (81), sphingolipid hydrolases (20), phosphoglyceride (189) and sphingolipid synthetases (135), sterol ester hydrolases and lipases (134). It is therefore recognized that interaction of lipid metabolizing enzymes with their substrates depends upon the chemical structure, as with all enzymes, but also in addition to this, the physical structure of their substrates (36). This physical structure can vary in size, in type of aggregate, and in electrostatic properties produced by the arrangement of polar groups on the outer surface of the aggregate (36). Detergents are able to alter these physical characteristics of a lipid substrate, by forming mixed micelles in combination with them. The charge properties and size of the mixed micelles formed will depend upon the nature of the detergent and its concentration relative to the lipid substrate (184).

It is difficult to separate the effect of detergent on substrate from the effects they have on the enzyme itself when dealing with lipid metabolizing enzymes. Further complications are introduced when the enzyme is membrane bound, since detergent effects on membrane structure may secondarily be reflected upon the enzyme activity. For these reasons cases of soluble enzymes acting on soluble substrates best illustrate the ability of certain enzymes to be activated by direct interaction with detergents.

The calmodulin regulated calcium dependent cyclic nucleotide phosphodiesterase (PDE) of brain is an example

of such a detergent activated enzyme (219,146). Sodium dodecyl sulfate, free fatty acids and lysophosphatidylcholine were able to activate the enzyme. What is particularly interesting is the suggestion that the detergent mode of action is to induce the activating conformational change in PDE usually caused by the calmodulin-calcium complex (219). Evidence for this is that, once activated by detergents, PDE can no longer be stimulated by calmodulin, nor does it show calcium dependence (219). Furthermore, the kinetic properties of PDE activated by either detergents or calmodulin-calcium were similar.

Some other examples of soluble enzymes activated by detergents are the following: Pyruvate oxidase of E. coli (30), phenylalanine hydroxylase of rat liver (50), glycogen phosphorylase kinase of rabbit skeletal muscle (181) and CTP-choline phosphate cytidylyltransferase of rat liver and lung (24,214). It seems that in certain cases, activation of soluble enzymes by detergents may reflect the fact that they are regulated enzymes. The PDE and glycogen phosphorylase kinase illustrate this point as both can be regulated by calmodulin (209) while the kinase is additionally regulated by phosphorylation-dephosphorylation (181). On the other hand, certain enzymes such as CTP:choline phosphate cytidylyltransferase which are activated by detergents have been postulated to be regulated by naturally occurring surfactant molecules in vivo (24,214).

During this study the possibility that one of these mechanisms of detergent activation applies to PLD has been examined.

3. MATERIALS AND METHODS

3.1 Animals and Preparation of Tissues

One month old Sprague Dawley rats were used throughout these studies and were maintained on a normal diet. They were sacrificed by decapitation immediately prior to use and the tissues were promptly removed and placed in cold 0.85% sodium chloride solution until processing.

The partial purification of phospholipase D and the preparation of tissue subcellular fractions are described in sections 3.8 and 3.12 respectively.

3.2 Lipid Extraction Procedures

Two lipid extraction procedures were used. The first, based on the method of Folch et al. (52), consisted of adding 20 vol. of chloroform/methanol (2:1, v/v) to the material to be extracted followed by vortex mixing with 0.2 vol. of 0.1 M KCl, followed six times with 0.4 vol. of chloroform/methanol/0.1 M KCl (3:47:48, v/v/v). Each resulting upper phase was discarded and the lower chloroform phase containing the lipids was retained.

The second lipid extraction procedure was based on the method of Bligh and Dyer (16). Chloroform and methanol were added to the aqueous solution being extracted to yield a one phase mixture with the composition of chloroform/methanol/water (1:2:0.8, v/v). Sufficient chloroform and 0.1 M KCl were added followed by vortex mixing to produce a two phase system having the composition chloroform/methanol/water (2:2:1.8, v/v). The final

chloroform phase contained the lipids.

3.3 Protein Determination

Protein was determined by the method of Peterson (144) using bovine serum albumin (Sigma Chem. Co.) as standard. This procedure is a modification of that of Lowry et al. (124) containing sodium dodecyl sulfate to avoid detergent and lipid interferences and to provide mild conditions for rapid solubilization of membrane proteins. In addition this technique contains an optional deoxycholate-trichloroacetic acid protein precipitation step for removal of interfering substances. This was necessary when assaying protein content in the presence of detergents, see section 4.1.1. The method of Warburg and Christian (210), based on ultraviolet absorption, was used for protein estimation of Sepharose 4B column fractions (section 3.8).

3.4 Phospholipid Phosphorous Determination

Phospholipid phosphorus was determined as described by Bartlett (8) using sodium phosphate (Sigma Chem. Co.) as a standard. This procedure is based upon the release of inorganic phosphate from phospholipids by acid digestion followed by the colorimetric determination of this phosphate as its reduced phosphomolybdate complex.

3.5 Determination of Radioactivity

Radioactivity was quantitated in a Searle Mark III

scintillation spectrophotometer using the commercially prepared scintillation fluid, Scintiverse (Fisher Sci. Co.). Efficiency was determined by the external standard method with a commercially prepared set of quenched liquid scintillation counting standards (Beckman Instruments, INC.). Quantitation of radioactivity on thin layer plates was carried out by scraping the silica gel into a counting vial, adding 0.5 ml of water and 10 ml of the scintillation fluid.

3.6 Preparation of Substrates for Phospholipase D Assay

3.6.1 Preparation of [2-^{3}H] glycerol substrate solutions

[2-^{3}H] Glycerol solutions at 2 M concentration and 2 Ci/mol specific activity were prepared by combining 1.0 ml of 2 M glycerol (spectroanalyzed grade, Fisher Sci. Co.) with 4.0 ml of [2-^{3}H] glycerol at 1 mCi/ml and 200 Ci/mol (as supplied by New England Nuclear) followed by evaporation at 45°C under a nitrogen flow to remove water. The glycerol was then made up to a final volume of 1.0 ml with double distilled water.

3.6.2 [^{3}H] Phosphatidylcholine preparation

1-Acyl-2-[9,10-^{3}H] oleoyl-sn-glycero-3-phosphoryl-choline was synthesized by a procedure based upon that described by Webster and Cooper (212). 50 μg (0.18 μmol) of oleic acid, 1050 μg (2.1 μmol) of 1-acyl-lysophosphatidylcholine (Serdary Research Lab.) 2 mCi of [9,10-^{3}H] oleic acid, 5.04 Ci/mmol (New England Nuclear) were

dried under nitrogen flow in a vial. Nitrogen is required because of the sensitivity of the double bond of oleic acid to oxidation. 250 μl of each of the following solutions was then added to the residue.

- 0.8 mM coenzyme A (P-L Biochemical Co.) in 2 mM β,β-dimethyl glutarate buffer, pH 3.8
- 80 mM ATP (P-L Biochemical Co.), pH 7.2
- 400 mM KH_2PO_4-K_2 HPO_4 buffer, pH 7.2
- 750 mM sodium flouride.

The mixture was vortex mixed and then placed for 15 minutes in a bath type sonicator (Heat-Systems-Ultrasonics Inc.). 1 ml of freshly prepared 16% w/v homogenate of rat liver in distilled water was added and the suspension was incubated for 1.5 hr at 37°C with shaking. The lipids were then extracted by the Bligh and Dyer procedure (sec. 3.2) and separated on a 20 x 20 cm silica gel G plate (Redi/plate, Fisher Sci. Co.) using procedure A described in section 3.10.1. Phosphatidylcholine was located on the thin layer chromatography plate by the whiter area produced as a result of water absorption by this lipid while the solvent dries after development. The use of iodine for locating this lipid was avoided in order to protect the double bonds present in esterified fatty acids (136). [^{3}H] phosphatidylcholine was recovered from this area of the thin layer plate by scraping the silica into a test tube and extracting it by the Bligh and Dyer method (sec. 3.2). Incorporation of [^{3}H] oleic

acid was greater than 90%. The specific radioactivity of the [^{3}H] phosphatidylcholine product was determined by the measurement of both radioactivity (sec. 3.5) and phospholipid phosphorous (sec. 3.4) to be 200 Ci/mol.

3.6.3 Preparation of phosphatidylcholine micro-dispersions

Radiolabeled phosphatidylcholine, [^{3}H] oleoyl phosphatidylcholine (sec. 3.6.2) or [U-^{14}C] phosphatidylcholine (1920 Ci/mol, New England Nuclear) and egg phosphatidylcholine, 50 μmole, (Serdary Res. Lab.) were dried under nitrogen flow in a 12 ml conical centrifuge tube. 2 ml of 5 mM β,β-dimethylglutarate buffer, pH 6.5, was added and the solution was sonicated under a continuous nitrogen flow using a probe type sonicator (Heat-Systems-Ultrasonics Inc., Model W185) set at 50 watts, for a total of 30 min, with the centrifuge tube in ice water. The sonication was carried out in six 5 min bursts spaced by 5 min time intervals to prevent heating of the phospholipid solution. The solution was centrifuged at 100,000 x g for 30 min to yield a supernatant solution which was used for phospholipase D assay. On the average 90% of the sonicated phosphatidylcholine was recovered in this supernatant solution.

3.7 Thin Layer Chromatography

Thin layer chromatography was carried out using either silica gel G (250 μ, Redi/plate, Fisher Sci. Co.) or HPTLC silica gel Merck 60 thin layer plates (Terochem

Laboratories) activated by heating at 110°C for 1 hour prior to use. Reagent grade solvents (Fisher Sci. Co.) were used for the solvent mixtures. Phospholipids were usually visualized on the silica gel thin layer plates by exposure to iodine vapor or in the case of [^{14}C] lipids by autoradiography. In this method the thin layer plates were placed in contact with Kodak "No Screen" x-ray film for 3 days after which the film was developed according to the manufacturer's directions. Phospholipid standards were purchased from Serdary Research Laboratories.

3.8 Partial Purification of Phospholipase D and Phosphatidylglycerol Forming Activities

Lyophilized rat brain homogenate was prepared as follows. One month old Sprague Dawley rats were killed by decapitation and the brains were removed. A 50% w/v homogenate in distilled water was prepared by homogenizing the brains in a Lourdes homogenizer (Vernitron Medical Products, Inc.) at 1/2 maximal speed for 4 minutes at 0°C. This material was lyophilized and stored at -20°C until required. 1.5 g of the lyophilized brain powder was resuspended in 40 ml of 5 mM Hepes, pH 8.0, with 5 passes in a Kontes glass homogenizer using a motor driven teflon grinder. This material was centrifuged at 100,000 x g for 30 min to yield a particulate fraction. Solubilization of this fraction was accomplished by rehomogenizing it in 160 ml of a solution containing 0.8% Miranol H_2M, 0.5% sodium

cholate and 5 mM Hepes buffer, pH 7.2. This material was centrifuged at 100,000 g for 30 min to yield a supernatant containing both solubilized phosphatidylglycerol forming activity and phospholipase D activity. The protein present in the solubilized extract was precipitated by adding ammonium sulfate to a 70% saturation, stirring for 30 minutes, and centrifuging at 29,000 x g for 30 min. The resulting pellet was resuspended to 10 ml in a solution which consisted of Miranol H_2M 0.25%, sodium cholate, 0.125%, 5 mM HEPES buffer, pH 7.2, 0.01%, β-mercaptoethanol and 1 mM EDTA. This solution was also used to elute the Sepharose 4B column.

A 10 ml aliquot of the solubilized concentrated solution was applied to a column of Sepharose 4B (2.5 x 70 cm) (Pharmacia) previously equilibrated with the Sepharose 4B elution buffer, and eluted. The fractions containing phosphatidylglycerol forming activity, tube numbers 13 to 19 (Fig. 1) were pooled and concentrated by ammonium sulfate precipitation as described above. The precipitate was then resuspended in a small volume of Sepharose 4B elution buffer and used as the enzyme source.

3.9 Assay Procedure for Phosphatidylglycerol Forming Activity and for Phospholipase D

3.9.1 Assay for phosphatidylglycerol formation in detergent solubilized membrane fractions

Phosphatidylglycerol formation was assayed by the incorporation of $[2\text{-}^3H]$ glycerol into phosphatidylglycerol in the presence of 0.8 μmol phosphatidylcholine and either 0.8 μmol cholate or 0.4 μmol taurodeoxycholate. Solvent solutions of the lipid and detergents were dried in the reaction tubes and then resuspended in 5 μmol β,β-dimethylglutarate, pH 6.5, 10 μl of 2.0 M glycerol solution, 2-4.6 Ci/mol and 10 μl, up to 200 μg of protein, of enzyme preparation were added to give a total final volume of 50 μl. After incubating at 37°C for 30 min the lipids were extracted according to the method of Folch (52) sec. 3.2. The phosphatidylglycerol formed was measured by scintillation counting after purification by chromatography on silica gel G in a solvent consisting of tetrahydrofuran/methylal/methanol/2 N ammonium hydroxide (50:40:10:5.5, v/v).

3.9.2 Assay for phosphatidylglycerol formation in intact membranes

Phosphatidylglycerol formation was assayed by the incorporation of $[2\text{-}^3H]$ glycerol into phosphatidylglycerol with microsomal membranes as the source of both the phospholipid substrate and phospholipase D. Sodium oleate was required as an activator in a ratio of 2 μmol/mg microsomal protein. The assay also contained 50 mM β,β-dimethylglutarate buffer, pH 6.5, 20 μmol of $[2\text{-}^3H]$ glycerol at 2-4.6 Ci/mol, and up to 200 μg of microsomal

protein in a reaction volume of 50 µl. The assay was incubated for 15 minutes at 30°C. The extraction and measurement of phosphatidylglycerol formed was as described in section 3.9.1.

3.9.3 Assay procedure for phospholipase D in intact membranes

Phospholipase D activity was assayed in the presence of 50 mM β,β-dimethylglutarate-NaOH buffer, pH 6.5, 5 mM EDTA, 50 mM potassium fluoride, 6 mM sodium taurodeoxycholate and 0.5 µmol of ^{3}H-oleoyl phosphatidylcholine microdispersion (1.94 Ci/mol). The reaction was initiated by adding up to 200 µg of protein and the final mixture, 100 µl total volume, was incubated at 30°C for 15 minutes. The reaction was terminated by adding 1.5 ml of chloroform-methanol 1:1 with carrier phosphatidic acid. The lipids were extracted by the Bligh and Dyer method and separated on HPTLC silica plates developed twice in the ascending direction. The first solvent mixture used was diethylether/acetic acid (100:1, v/v), followed by chloroform/acetone/acetic acid/methanol/water (50:20:10:10:5, v/v). The PA areas were measured for radioactivity by scraping and counting the silica directly.

3.9.4 Assay for phospholipase D in detergent solubilized membrane fractions

Phospholipase D activity was measured in the presence of 20 µl of a 20 mM microdispersion of [U-^{14}C] phosphatidylcholine (1000 cpm/nmol), 0.2 µmol taurodeoxycholate, 10 µmol β,β-dimethylglutarate, pH 6.5, and 20 µl of

enzyme preparation in a final volume of 100 μl. Where indicated, phosphatidylglycerol formation was assayed under the same conditions as given above for phospholipase D, with [U-^{14}C] phosphatidylcholine as substrate, by the addition of 43 μmol of unlabeled glycerol to the incubations. After reaction for 30 minutes at 37°C the lipids were extracted according to the procedure of Bligh and Dyer, sec. 3.2, and separated by two dimensional TLC in solvent system A, sec. 2.10.1.

3.10 Identification of Phosphatidylglycerol Formed by Brain Extract

3.10.1 Chromatographic procedures

Three separate two dimensional silica gel thin layer chromatography procedures were used for this purpose.

Procedure A, Rouser et al. (164), employed chloroform/methanol/concentrated ammonia (65:35:5, v/v) in the first direction followed by chloroform/acetone/acetic acid/methanol/water (50:20:10:10:5, v/v) in the second direction with silica gel G plates.

Procedure B, Poorthius et al., (148) employed chloroform/methanol/water/concentrated ammonia (70:30:3.2, v/v) in the first direction and chloroform/methanol/water (65:25:5, v/v) in the second direction with silica gel G silica plates pre-sprayed with a 0.4 M boric acid solution before activation at 110°C.

Procedure C, Hallman and Gluck, (73) employed tetrahydrofuran/methylal/methanol/2 N ammonia (50:40:10:5.5,

v/v) in the first direction followed by chloroform/methanol/concentrated ammonia (65:25:5, v/v) in the second direction with silica gel G plates.

The water soluble products of alkaline and acetic acid hydrolysis were separated by descending chromatography with Whatman No. 1 paper and 2-propanol/water/concentrated ammonia (7:2:1, v/v) as solvent (37).

3.10.2 Alkaline hydrolysis of phospholipids

Phospholipids were subjected to alkaline hydrolysis treatment according to the procedure of Davidson and Stanacev (33). Chloroform/methanol (2:1, v/v) solutions containing 1.0 mg of phosphatidylglycerol (Serdary) and 98,000 dpm of the lipid were dried under nitrogen flow. The dried lipid was dissolved in 2.5 ml of chloroform/methanol (1:1, v/v) and 50 µl of 4 N NaOH. The reaction was allowed to proceed for 10 minutes at room temperature after which 250 mg of Dowex 50W-X8 cation exchange resin (^{+}H) form was added with mixing. The mixture was extracted with 1 ml of water and centrifuged to separate phases. This extraction was repeated twice, the aqueous phases combined, made alkaline by adding 10% NH_4OH solution and then evaporated to dryness under nitrogen flow. The residue was made up in a small volume of water for application to paper chromatograms.

3.10.3 Acetic acid hydrolysis of phospholipids

Phospholipids were subjected to acetic acid hydrolysis according to the procedure of Yang et al. (226). Chloroform/methanol (2:1, v/v) solutions containing 1.0 mg of phospha-

tidylglycerol (Serdary) and 98,000 dpm of the lipid were dried under nitrogen flow. The dried lipid was resuspended in 1.0 ml of 85% v/v acetic acid with mixing and placed in a boiling water bath for 15 min. The acetic acid was evaporated with nitrogen flow at 35°C and 2 ml each of water and diethylether were added with mixing. Phases were separated with centrifugation and the aqueous layer removed, evaporated to dryness with nitrogen flow and made up in a small volume of water for application to paper chromatograms.

3.10.4 Preparation of isopropylidene derivative of phosphatidylglycerol

A modification of a published procedure was used (152) 50 µg of phosphatidylglycerol and 80,000 dpm of the lipid were dried with nitrogen flow in a screw cap vial. 1 ml of anhydrous zinc chloride/acetone 25% w/w solution was added with mixing and the solution shaken at 37°C for 4.5 hr. The reaction was stopped by adding 5 ml of chloroform/methanol (2:1, v/v) and then extracted by the Folch method, to yield a final chloroform phase. This chloroform extract was dried and then applied to an activated silica gel G plate. The plates were developed in chloroform/methanol/concentrated ammonia (65:25:5, v/v) and the isopropylidene derivative, R_f = 0.8 was well separated from phosphatidylglycerol, R_f = 0.50. A reaction yield of about 90% was estimated from the recovery of radioactivity in the isopropylidene area of the thin layer plate. The derivative was recovered from the thin

layer plate by scraping the silica gel from the appropriate area and extracting by the procedure of Bligh and Dyer. The isopropylidene phosphatidylglycerol, 50 μg, 3500 cpm was hydrolyzed by adding 0.5 ml of 0.5 M HCl to a test tube containing the dried derivative, and shaking the mixture at 37°C for 20 min. The lipids were re-extracted and chromatographed as described above adding carrier phosphatidylglycerol. The yield for hydrolysis of the derivative to produce phosphatidylglycerol was estimated to be 50% based upon the recovery of radioactivity as phosphatidylglycerol.

3.10.5 Phospholipase C treatment of phospholipids

The method of Haverkate and Van Deenen (77) was used. The labeled lipid, 20,000 dpm, and 150 μg of standard phosphatidylglycerol were mixed and dried in a conical centrifuge tube with nitrogen flow. The lipids were dissolved in 300 μl of diethylether, 300 μl of 200 mM Tris-HCl buffer, pH 7.4, 300 μl of 20 mM $CaCl_2$, 35 μl of phospholipase C solution (Sigma Chem. Co., Type III from Bacillus cereus, 0.88 mg/ml), and the mixture was incubated at 37°C for 12 hr. The lipids present after the Folch extraction procedure were examined by TLC on silica gel G with hexane/ether (60:40, v/v) as the solvent. The water soluble material liberated in the case of ^3H labeled product, was examined by TLC chromatography on cellulose plates (Analtech Inc., MW300 normal cellulose, 100 μ thick), with n-butanol/acetic acid/water (65:25:5,

v/v) as solvent.

3.10.6 Phospholipase D treatment of phospholipids

A sample containing 20,000 dpm of the reaction product formed from [U-^{14}C] phosphatidylcholine was combined with 500 μg of standard phosphatidylglycerol to the method of Kates and Sastry (110). Lipids were dried in a 12 ml conical tube and then dissolved in 200 μl of 0.2 M acetate buffer, pH 5.6, 40 μl of 1 M $CaCl_2$, 200 μl of 20 mg/ml phospholipase D solution, (Boehringer-Mannheim, cabbage) and 160 μl of diethyl ether. The mixture was shaken at room temperature for 3 hr after which the lipids were extracted by the procedure of Bligh and Dyer (sec. 3.2) and separated by thin layer chromatography on silica gel G in the solvent system chloroform/methanol/concentrated ammonia (65:25:5, v/v).

3.10.7 Determination of the stereochemistry of the phosphatidylglycerol formed

The previously published method of Bublitz and Kennedy (22) was modified for the determination by radiochemical analysis rather then by spectrophotometry. [^{3}H] glycerol-3-phosphate liberated by phospholipase C treatment of the [^{3}H] phosphatidylglycerol produced by the rat brain enzyme, was purified on cellulose TLC in the solvent n-butanol/acetic acid/water (50:30:10, v/v), eluted from the cellulose with water and concentrated under N_2 flow. Incubation of the extracted glycerol phosphate with sn-glycerol 3-phosphate dehydrogenase was as follows. The material was dried under N_2 in test tubes, to which were

added 40 µl of 5 mM β-NAD (Sigma Chem. Co.), 20 µl of 400 mM hydrazine, pH 9.5 and 2 µl of sn-glycerol 3-phosphate dehydrogenase (Boehringer-Mannheim, rabbit muscle). The mixture was incubated at 37°C for 15 min after which the reaction mixture along with carrier GP and dihydroxyacetone-phosphate (DHAP) were applied directly to cellulose plates and developed in the same solvent given above. Quantitation of the radioactivity corresponding to glycerol 3-phosphate, R_f = 0.17 and to NADH, R_f = 0.00 was by scintillation counting of the appropriate areas of the plates.

Spectrophotometric verification of the radiochemical sn-glycerol 3-phosphate determination was carried out as follows. The same procedure was used except that 10 nmol of L-α-glycerol-3-phosphate was added to one tube and 10 nmol of DL-α-glycerol-3-phosphate was added to another. Following reaction, the assays were diluted with 400 µl of ice cold water and absorbance was determined at 340 nm. The percent of reaction was calculated based upon an extinction coefficient for β-NADH of 6.22×10^6 cm^2/mole at 340 nm.

3.11 Preparation of Subcellular Fractions of Rat Tissue

The preparation of subcellular fractions was similar to the procedure described by Cotman and Matthews (28).

3.11.1 Microsomal fractions from rat organs

Four male Sprague Dawley rats, one month old, were decapitated and the organs removed immediately to ice cold 0.85% NaCl solution. 10% w/v homogenates of organs

in 0.32 M sucrose were prepared by first mincing with scissors and then homogenizing with a glass homogenizer and motor driven teflon pestle. The homogenates were centrifuged 17,000 g x 10 minutes to yield a supernatant which was then centrifuged 100,000 g x 60 minutes to yield a microsomal pellet. The microsomes were resuspended with 10 passes of a Dounce homogenizer with tight fitting glass pestle.

3.11.2 Subcellular fractions of brain and lung

Homogenates (10% w/v) were centrifuged 200 g x 5 min in a Sorvall HB4 swinging bucket rotor to sediment unbroken cells and large debris. The supernatant (H) was centrifuged 1100 g x 5 min to yield a pellet (P_1) the nuclear fraction. The P_1 pellet was washed by rehomogenizing in 25 ml of 0.32 M sucrose and resedimented at 1100 g x 5 min. The combined supernatants were then centrifuged at 17,000 g x 10 min to yield a pellet (P_2) the mitochondrial fraction, which was washed as described for the nuclear fraction. The combined supernatants were again centrifuged at 17,000 x g for 10 min to yield a pellet P2-B the microsomal wash. The supernatant was centrifuged 100,000 x g for 90 minutes to yield a pellet P_3, the microsomal fraction and a supernatant, S, the cytosolic fraction. The microsomal fraction was resuspended in 0.32 M sucrose with 10 passes of a tightly fitting Dounce homogenizer.

3.12 Assay of NADPH-cytochrome C Reductase

NADPH-cytochrome c reductase was measured according to the procedure of Sottocasa et al. (187) using a recording spectrophotometer.

3.13 Treatment of Microsomal Membranes With Proteases and Mercurial Reagents

Proteases, nonpenetrating mercurial reagents and deoxycholate treatments for establishing the sidedness of the microsomal membrane phospholipase D were similar to those of Dallner et al. (139).

Protease treatment

Freshly prepared microsomal fraction (3.5 mg) was incubated in a total volume of 1.0 ml in the presence of 50 mM Tris pH 7.5, 50 mM KCl, 0.32 M sucrose, 350 μg of protease in the presence or absence of 0.05% w/v deoxycholate. Incubations were for 15 min at 30°C and were initiated by the addition of the microsomes. The reactions were stopped by adding 9.0 ml of ice cold 0.32 M sucrose and 700 μg of trypsin inhibitor to experiments with trypsin. Microsomal membranes were sedimented by centrifugation at 100,000 x g for 60 minutes and after discarding the supernatant, were resuspended in 500 μl of 0.32 M sucrose and assayed for phospholipase D activity.

Parachloromercuriphenylsulfonic acid treatment was similar except that the reaction contained 50 mM β-β-dimethylglutarate and up to 600 μM PCMPS. Incubations were

for 30 minutes on ice after which they were stopped by the addition of 24 ml of ice cold 0.32 M sucrose. Other details were as for protease treatment.

4. RESULTS

The results are presented in three sections. The first describes a novel phosphatidylglycerol (PG) forming activity in rat brain detergent solubilized membranes. The production of this phospholipid was demonstrated by analytical methods and the enzyme involved was characterized. A comparison of this activity and phospholipase D (PLD) also present in the detergent extracts was carried out and provided evidence for the likely identity of these two enzymes (sec. 4.1). Properties of the PLD present in intact rat brain membranes are reported in section 4.2. Both PG forming activity, and hydrolytic activity were used to assay this enzyme in subcellular fractions and in various tissues. Proteases and the nonpenetrating inhibitor compound PCMPS were used to establish the sidedness of PLD on microsomal membranes. PLD was found to be undetectable in microsomes when assayed without an activator such as taurodeoxycholate. The results of a detailed study of the latency displayed by PLD are presented in sec. 4.3. The PG forming activity was primarily used for this study because it measures the in situ activity of PLD, towards the endogenous phospholipids of the microsomal membrane. While latency towards exogenous substrates is of interest it may not involve a physiologically occurring situation. Latency towards the endogenous membrane lipids does suggest that this phenomenon reflects a true property of the enzyme in vivo. A range of naturally occurring amphiphilic lipids were

tested for their ability to activate PLD. Besides the bile salts unsaturated fatty acids were found to be effective activators. Some properties of the fatty acid activated PLD are reported in sec. 4.3.

4.1 Characterization of a Phosphatidylglycerol (PG) Forming Activity of Rat Tissue

4.1.1 Preparation of PG forming enzyme containing fraction

The PG forming enzyme activity was initially observed in a detergent solubilized membrane fraction of rat brain (sec. 3.8). A search for this activity in intact membranes revealed that negligible quantities could be detected. Later results demonstrate that a high degree of latency is displayed by the PG forming and by the PLD enzyme in the absence of detergent activator. This necessitated the subsequent use of solubilized membranes for these studies (sec. 4.1). The specific activity of PG formation was 9 $nmol.mg^{-1}.hr^{-1}$ in the ammonium sulfate concentrated fraction and 18 $nmol.mg^{-1}.hr^{-1}$ in the combined active fractions recovered from the Sepharose 4B column, tubes 16 to 25 (Fig. 1). The product formed by this partially purified enzyme was separated by thin layer chromatography, extracted from the silica and then subjected to analysis in order to identify it. These results are now described.

4.1.2 Identification of phosphatidylglycerol

4.1.2.1 Thin layer chromatography

In each of three separate two dimensional silica gel

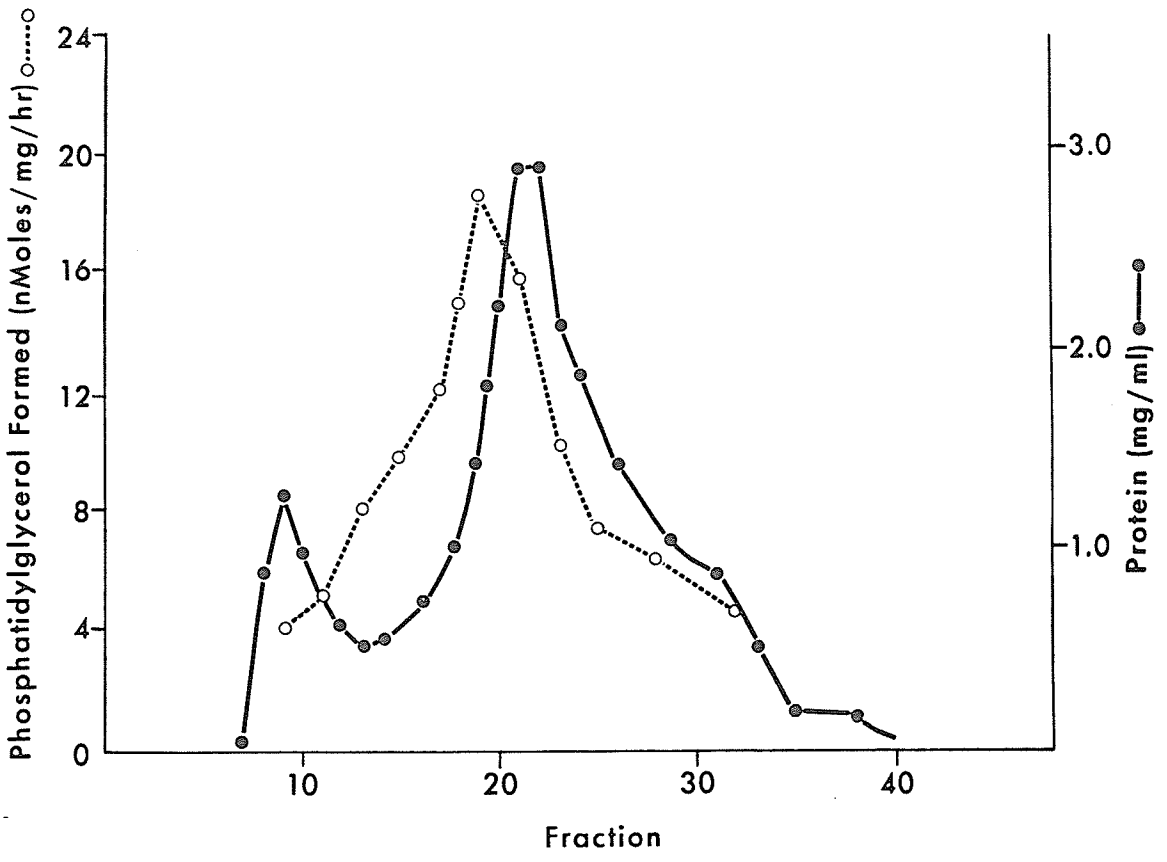


Figure 1. Sepharose 4B column chromatography of the phosphatidylglycerol forming activity. A 30 μl portion of each column fraction was taken and the enzyme activity assayed as described in sec. 3.9.1.

thin layer chromatographic solvent systems the radioactive product formed in the presence of [U-^{14}C] phosphatidylcholine and unlabeled glycerol was found to co-chromatograph with authentic phosphatidylglycerol (Table 1). This suggestive evidence that the compound was PG therefore warranted further proof.

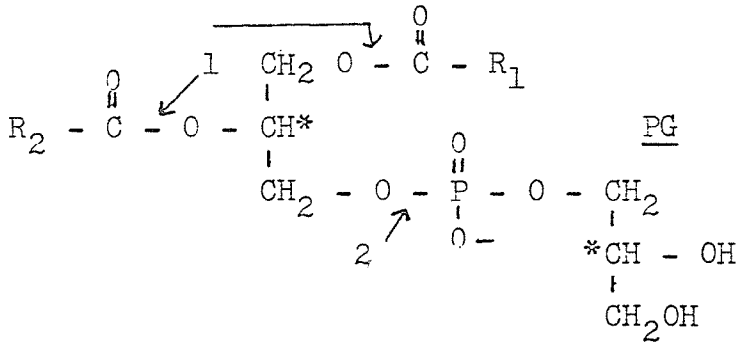
4.1.2.2 Chemical derivatization

The results of three different chemical derivatizations of the lipid product and of standard PG are shown in Table 2, and Scheme 1. The lipid prepared from [2-^{3}H] glycerol and unlabeled phosphatidylcholine, yielded products identical with those produced from the standard PG after the same treatment. Co-chromatography of these reaction products along with standard compounds when possible, was used to indicate their identity.

4.1.2.2.1 Mild alkaline hydrolysis of PG removes the fatty acid esters and results in the water soluble product, glycerol phosphorylglycerol (Scheme 1). This product co-chromatogramed on paper with the radioactive water soluble product from the lipid.

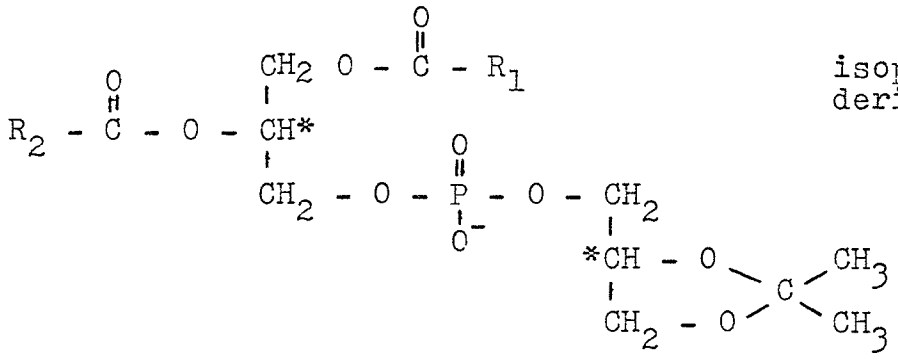
4.1.2.2.2 The water-soluble material produced by acetic acid hydrolysis of the lipid (Scheme 1) had an R_f identical to that obtained from treatment of standard PG and to authentic sn-glycerol 3-phosphate, indicating that the radioactive glycerol in the isolated PG possesses a free hydroxyl group which is vicinal to a phosphate diester linkage (29).

Scheme 1 Structural Analysis of PG

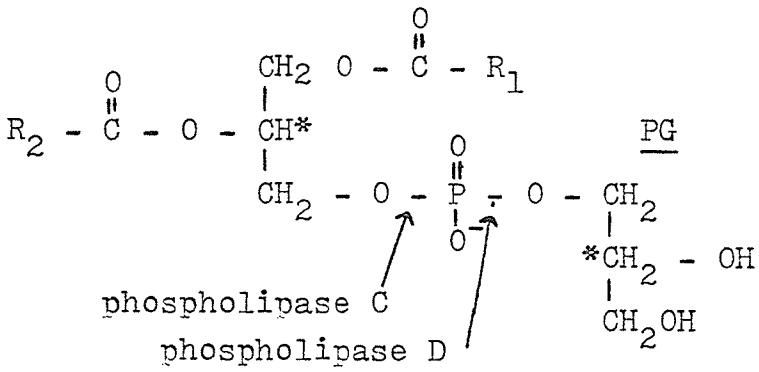


1 mild alkaline hydrolysis

2 acetic acid hydrolysis



isopropylidene derivative of PG



Asterisks indicate asymetric carbons of PG

TABLE 1

Phosphatidylglycerol identification by thin layer chromatography

	Solvent systems	R_f	
		Product	Authentic PG
A)[1]	$CHCl_3$-MEOH-NH_4OH (65:35:5) (v/v)	0.61	0.61
	$CHCl_3$-ACETONE-CH_3COOH-MEOH-H_2O (50:20:10:10:5) (v/v)	0.54	0.54
B)[2]	$CHCl_3$-MEOH-H_2O-NH_4OH (70:30:3:2) (v/v)	0.37	0.37
	$CHCl_3$-MEOH-H_2O (65:25:5) (v/v)	0.87	0.87
C)[1]	THF-METHYLAL-MEOH-2N-NH_4OH (50:40:10:5) (v/v)	0.30	0.30
	$CHCl_3$-MEOH-NH_4OH (65:35:5) (v/v)	0.45	0.45

1. Silica gel G plates were employed.

2. Boric acid impregnated silica gel G plates were employed.

4.1.2.2.3 The ability of the PG produced by the rat brain fraction to form an isopropylidene derivative (Scheme 1) requires the presence of two vicinal hydroxyl groups (152) and therefore indicates that the phosphate is linked to the terminal carbon of the unesterified glycerol moeity. The isopropylidene derivative of the in vitro product upon aqueous hydrolysis regenerated the parent compound. Authentic phosphatidylglycerol subjected to this treatment gave identical results (Table 2).

The ability of a number of phospholipases to hydrolyze the lipid product provided additional evidence identifying it as phosphatidylglycerol.

4.1.2.3 Phospholipase degradation

The lipid product obtained from $\left[U\text{-}^{14}C\right]$ phosphatidylcholine and samples of standard PG were incubated separately with phospholipases C and D (Scheme 1) and the hydrolysates analysed by thin layer chromatography (Table 3). Phospholipase D treatment gave a labeled material having an R_f identical to that of standard phosphatidic acid and to the material from standard phosphatidylglycerol. Similarly, phospholipase C hydrolysis produces a material having an R_f identical to that of authentic 1,2-diglyceride, the expected product from phosphatidylglycerol standard.

4.1.2.4 Stereochemistry of the phosphatidylglycerol produced by the rat brain fraction

As shown in Scheme 1, phosphatidylglycerol contains 2 asymetric centers, and so can exist as 4 separate

TABLE 2

Identification of chemical derivatives of phosphatidyl-glycerol by thin layer chromatography

	Treatment	R_f	
		Derivative	Authentic PG
(A)	Mild Alkaline Hydrolysis[1]	0.36	0.36
(B)	Acetic Acid Hydrolysis[1]	0.12	0.12
(C)	Isopropylidene Formation[2]	0.80	0.80

1. Paper chromatography with 2-PROPANOL-H_2O-NH_4OH (7:2:1) (v/v) as solvent.
2. TLC on silica gel G plates with $CHCl_3$MEOH-NH_4OH (65:35:5) (v/v) as solvent.

TABLE 3

Results of thin layer chromatography examination of phospholipase digests of phosphatidylglycerol

	Biosynthetic PG	Authentic PG	Authentic PA	Authentic diglyceride
Phospholipase D[1]	0.15	0.15	0.15	--
Phospholipase C[2]	0.44	0.44	--	0.44

1. Separation was accomplished on TLC silica gel G plates with $CHCl_3$-MEOH-14.8 N NH_4OH (65:25:4 v/v) as solvent.
2. Separation was accomplished on TLC silica gel G plates with hexane-ether (60:40 v/v) as solvent.

stereochemical isomers. The PG produced via the de novo pathway has been shown (77,33) to be of one isomeric form only, which is 3-sn-diacylglycerophospho-1^1-,sn-glycerol. The PG formed by plant PLD by a direct exchange was found to be different since it contained a racemic mixture of two isomers 3-sn-diacylglycerophospho-$1^1,3^1$-sn-glycerol (98). The $[^3H]$glycerol 3-phosphate liberated by phospholipase C from phosphatidylglycerol, produced from $[2\text{-}^3H]$glycerol and unlabeled phosphatidylcholine, was incubated with the stereospecific enzyme sn-glycerol 3-phosphate dehydrogenase (sec. 3.10.7). Approximately 35% of the ^3H was lost from glycerol 3-phosphate and appeared in NADH produced (Table 4). This suggests that the enzyme present in these rat brain extracts produces a racemic mixture of 3-sn-diacylglycerophospho-$1^1,3^1$-sn-glycerol.

Taken together, the results from all of these analytical techniques indicate the presence of a phosphatidylglycerol producing enzyme activity in rat brain. The characteristics of this enzyme were examined with the intent of explaining whether the previously described de novo pathway for this lipid (111) could be involved. On the other hand, if glycerol is incorporated by a direct exchange, it would be reasonable to suspect that perhaps one of the previously described phospholipid head group exchange enzymes, (44,102) perhaps rat brain phospholipase D, (169) or perhaps a novel glycerol exchange enzyme may be involved.

TABLE 4

Steriochemistry of the glycerol 3-phosphate liberated by phospholipase C treatment of phosphatidylglycerol

Treatment	Glycerolphosphate (cpm)	NADH (cpm)
Control	2834	55
Experimental	1894	968

The material liberated by phospholipase C was incubated with sn-glycerol 3-phosphate dehydrogenase and the incubation mixture applied directly to cellulose thin layer plates. The radioactivity associated with NADH and glycerophosphate was determined. The results are the average of two analyses.

4.1.3 Properties of the enzyme producing phosphatidylglycerol

4.1.3.1 Effect of time and protein concentration

The reaction was found to be linear for approximately 15 min (Fig. 2) and with up to about 4 mg/ml of the partially purified protein (Fig. 3).

4.1.3.2 The effect of incubation mixture pH on phosphatidylglycerol forming activity

The phosphatidylglycerol forming activity was found to be optimal at a pH of 6.0 (Fig. 4). Significant activities of the enzyme were observable at other pH values tested.

4.1.3.3 The effect of divalent cations and chelating agents on the activity

The base exchange activities have an absolute requirement for calcium ions (101), while phospholipase D does not (169). It was therefore of interest to determine whether any cation requirements exist for this phosphatidylglycerol formation. The presence of up to 50 mM of the cation chelators ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis-(β-aminoethyl ether) N,N^1-tetra acetic acid (EGTA) in the incubation mixture did not cause an inhibition of the activity, thus indicating a lack of divalent cation requirement. The addition of a range of divalent cations as their chlorides to the incubation was examined (Fig. 5). A general inhibitory effect of these ions was noted. Zinc and manganese were particularly effective inhibitors, while calcium, magnesium and barium

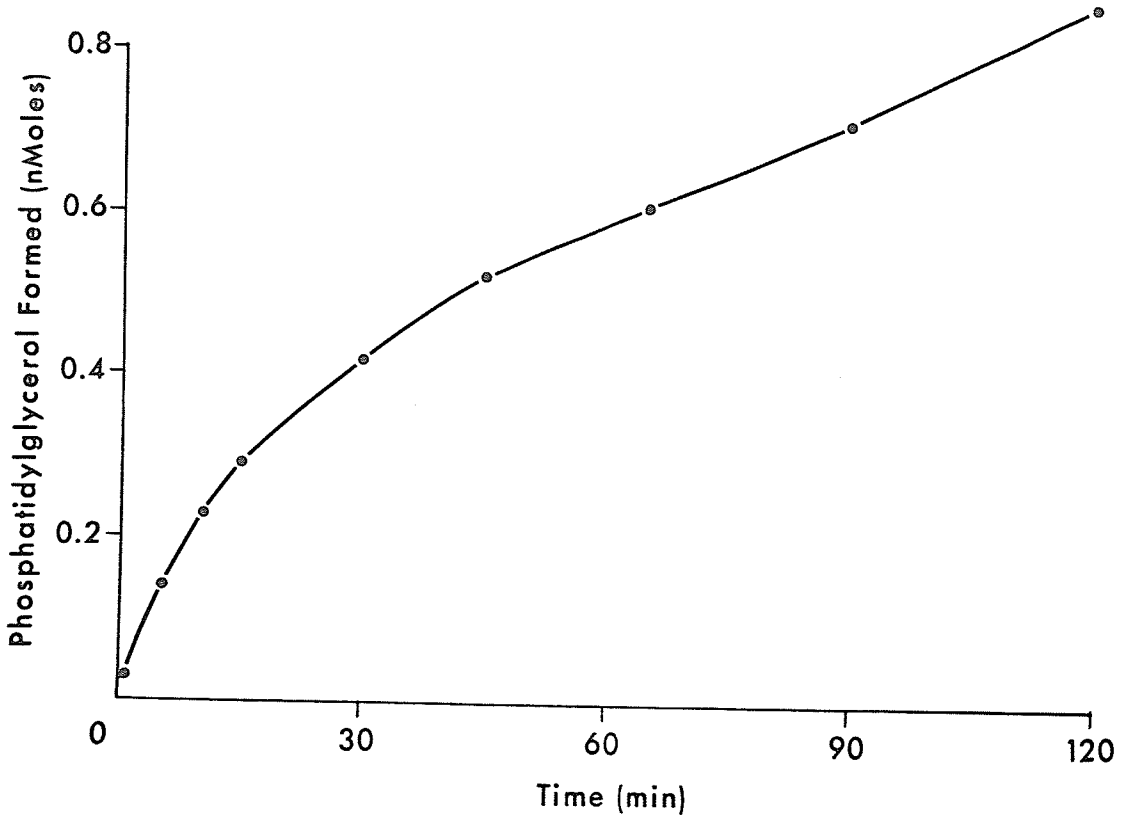


Figure 2. The effect of time on phosphatidylglycerol formation. The combine active fraction, tube No. 16 to 25, was assayed for various times as described in sec. 3.9.1.

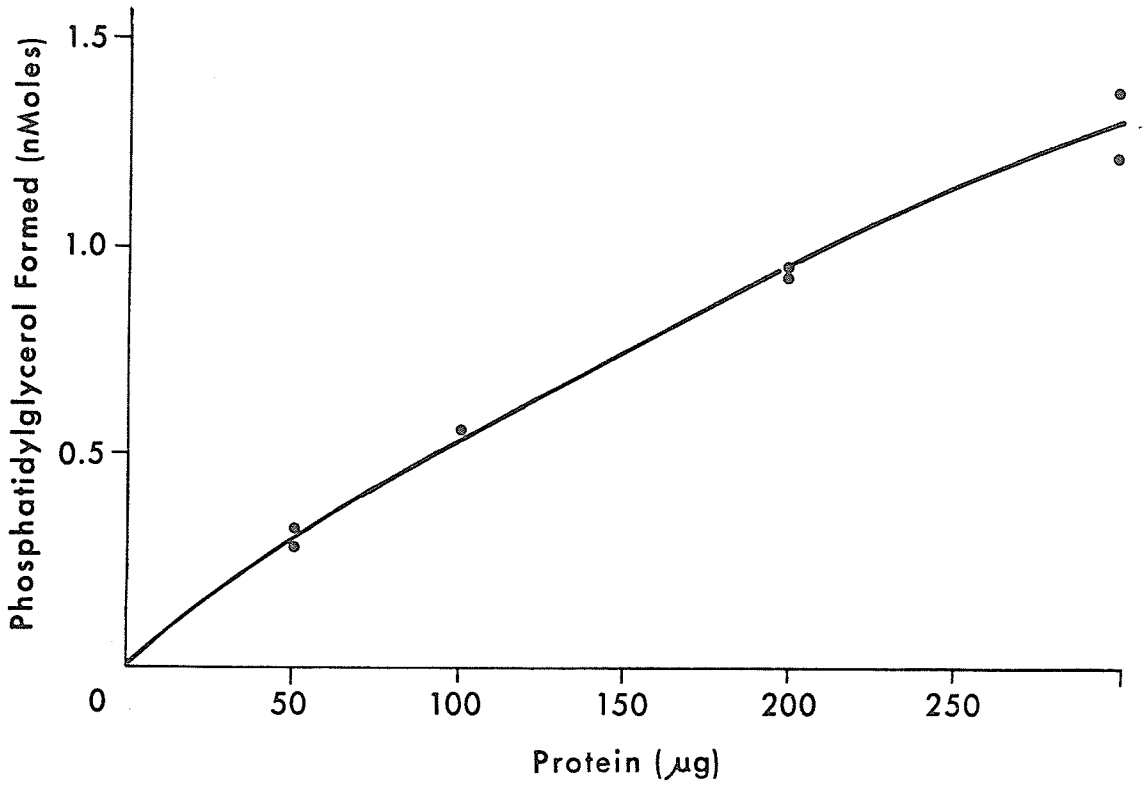


Figure 3. The effect of protein concentration on phosphatidylglycerol formation. The indicated amounts of the Sepharose 4B partially purified enzyme was assayed as described in sec. 3.9.1.

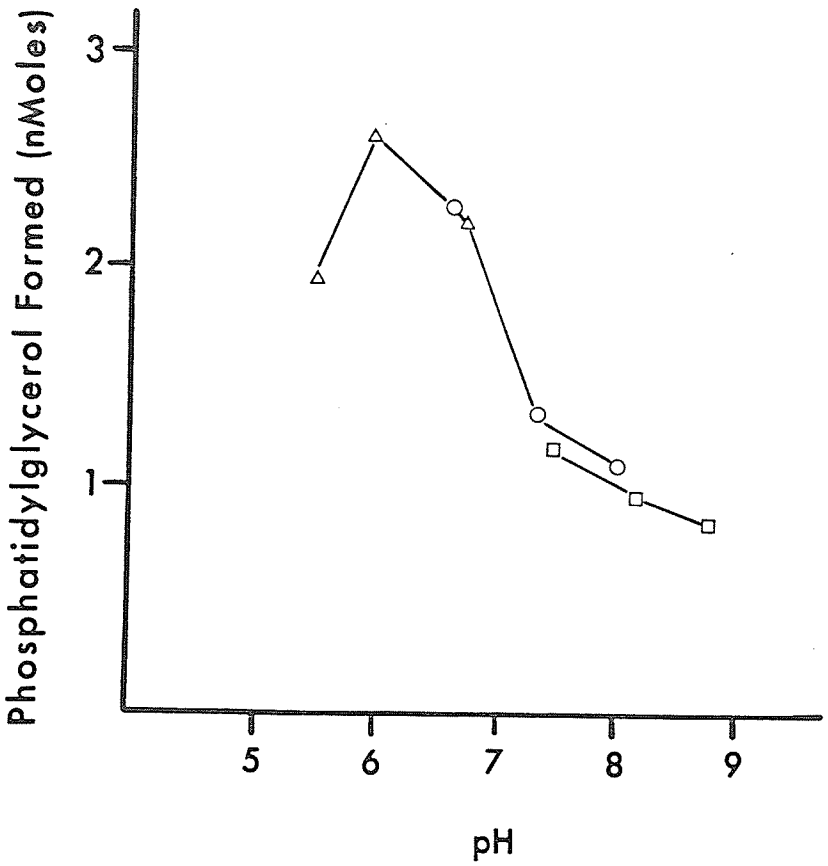


Figure 4. The variation of phosphatidylglycerol forming activity with pH of incubation mixture. Assay procedure was as described in sec. 3.9.1. Δ , MES buffer; o, HEPES buffer; □ , glycylglycine buffer.

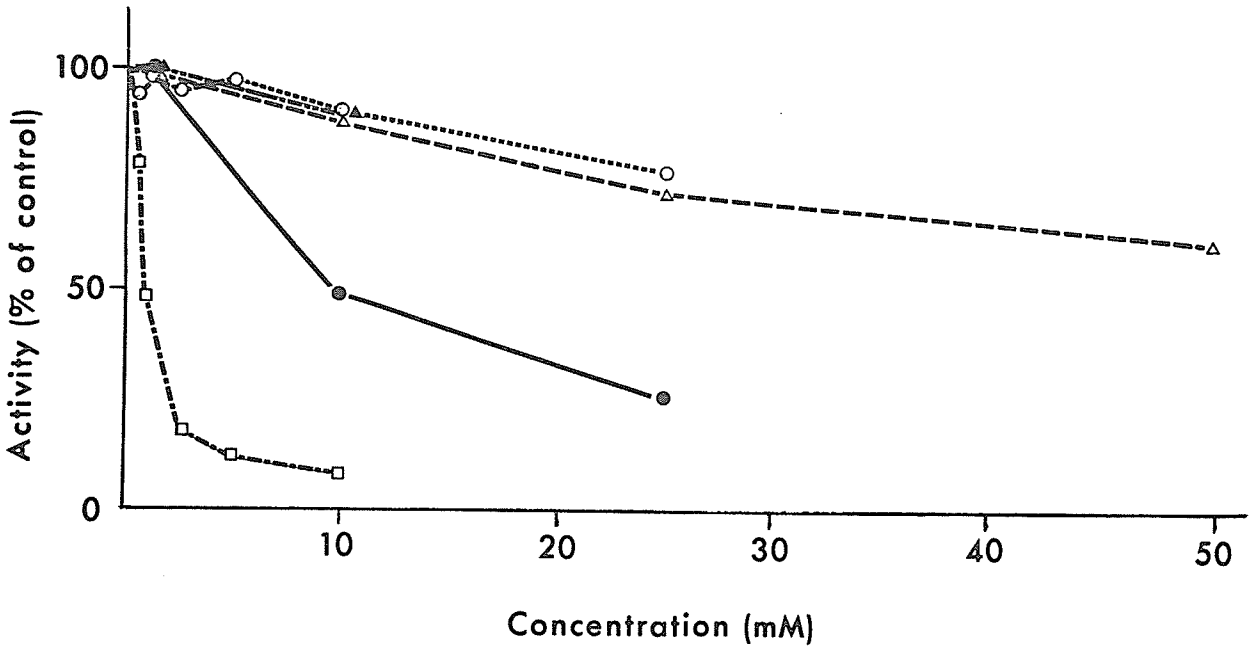


Figure 5. The effect of various cations on the formation of phosphatidylglycerol by the partially purified enzyme. Assays were as described in sec. 3.9.1 in the presence of chloride salts of the cations: ○ , Ba^{2} ;△ , Ca^{2} ;▲ , Mg^{2} ; ●, Mn^{2} ;□, Zn^{2} .

inhibited only at higher concentrations.

4.1.3.4 Effect of detergents on phosphatidylglycerol forming activity

The activity of many enzymes acting on lipids is modified by the presence of detergents in the assay mixture. Experiments to determine if this was also the case for the PG forming enzyme were carried out. With a 16 mM final concentration of an egg phosphatidylcholine microdispersion (sec. 3.6.3) very low activity was detected in the absence of added detergent. A maximum stimulation of the activity was observed with the addition of 8 mM taurodeoxycholate to the assay (Table 5). Other bile salts were also effective in stimulating the activity, while a variety of other detergents were found to have only a slight effect.

4.1.3.5 Phospholipid substrates for phosphatidylglycerol formation

A range of phospholipids was examined as possible substrates for phosphatidylglycerol formation. When each was added to the assay system in a 2:1 molar ratio with taurodeoxycholate it was apparent that phosphatidylcholine was the most effective substrate (Table 6). Phosphatidylglycerol and asolectin were much less effective as substrates. The remaining phospholipids tested were inactive.

There was also a phosphatidylglycerol-dependent incorporation of labeled choline into material co-chromatographing with phosphatidylcholine, suggesting that this is

TABLE 5

Effect of detergents on phosphatidylglycerol formation

	Detergent mM	Phosphatidylglycerol formed (nmoles)
No addition	—	0.05
Taurodeoxycholate	16	2.13
	8	4.10
Deoxycholate	16	2.49
	8	1.79
Glycodeoxycholate	16	1.12
	8	2.59
Cholate	16	0.81
	8	0.69
	3.2	0.20
Taurocholate	16	1.00
	8	1.60
Sodium dodecyl sulfate	16	0.25
	8	0.49
	3.2	0.20
Triton X-100	8	0.25
	3.2	0.12

Experiment conducted in presence of 16 mM phosphatidylcholine sonicated 1 hour at 60W in a Heat-Systems-Ultrasonics Inc. probe-type sonicator. Either 0.8 or 0.4 μmoles of the detergents in ethanol were combined with 0.8 μmoles of egg phosphatidylcholine in chloroform and dried under nitrogen flow in the test tubes.

TABLE 6

Ability of various phospholipids to act as substrate for phosphatidylglycerol formation

Phospholipid (15 mM)	Phosphatidylglycerol formed (nmoles)
No added phospholipid	0.00
Phosphatidylcholine	3.80
Phosphatidylsulfocholine	3.50
Phosphatidic acid	0.00
Phosphatidylethanolamine	0.12
Phosphatidylserine	0.01
Phosphatidylinositol	0.03
Phosphatidylglycerol	0.47
Asolectin	1.09
Phosphatidylethanolamine plasmalogen	0.12
Sphingomyelin	0.00

Further details of the assay are given in sec. 3.9.1.

a reversible reaction. Phosphatidylsulfocholine was observed to be nearly as effective a substrate as phosphatidylcholine, indicating that the nitrogen of choline is not essential for the recognition of the substrate by this enzyme.

The effect of various concentrations of phosphatidylcholine on the formation of phosphatidylglycerol was examined. A constant 2:1 molar ratio of phosphatidylcholine to taurodeoxycholate was maintained in the incubation (inset, Fig. 6). Saturation of the enzyme with this substrate occurred at about a 15 mM concentration. An apparent K_m of 3.5 mM was calculated from the Lineweaver-Burk plot of these data (Fig. 6).

4.1.3.6 The effect of glycerol

The effect of various glycerol concentrations in the incubation system was examined. Maximum activity was seen at about 0.5 M glycerol (inset, Fig. 7). A Lineweaver-Burk plot of these data indicated an apparent K_m of 0.2 M for this substrate (Fig. 7).

4.1.4 Comparison of the phosphatidylglycerol forming enzyme activity with phospholipase D

The properties of the phosphatidylglycerol forming reaction suggested that it may be catalysed by phospholipase D. The strongest evidence in support of this was its lack of calcium dependence and the low affinity observed for glycerol. The following series of experiments were designed to provide a direct comparison of the enzyme(s) responsible for phospholipase D and for phosphatidylglycerol

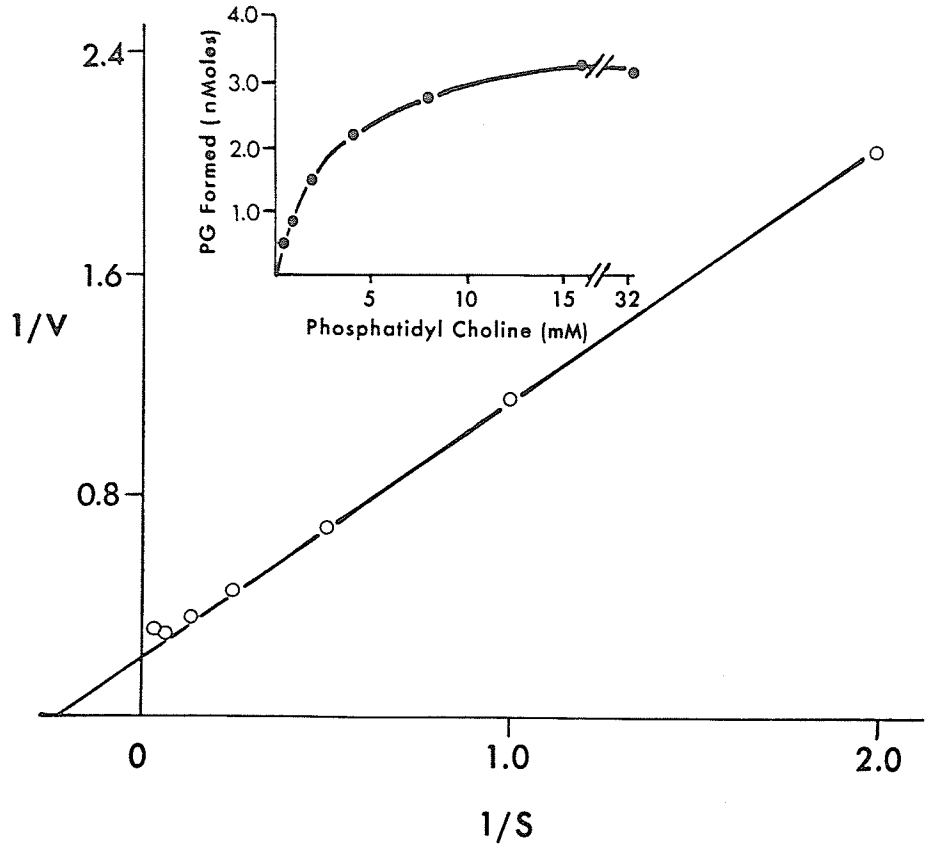


Figure 6. The effect of phosphatidylcholine concentration on phosphatidylglycerol formation. Assayed as described in sec. 3.9.2 in the presence of a constant 2:1 molar ratio of phosphatidylcholine to taurodeoxycholate.

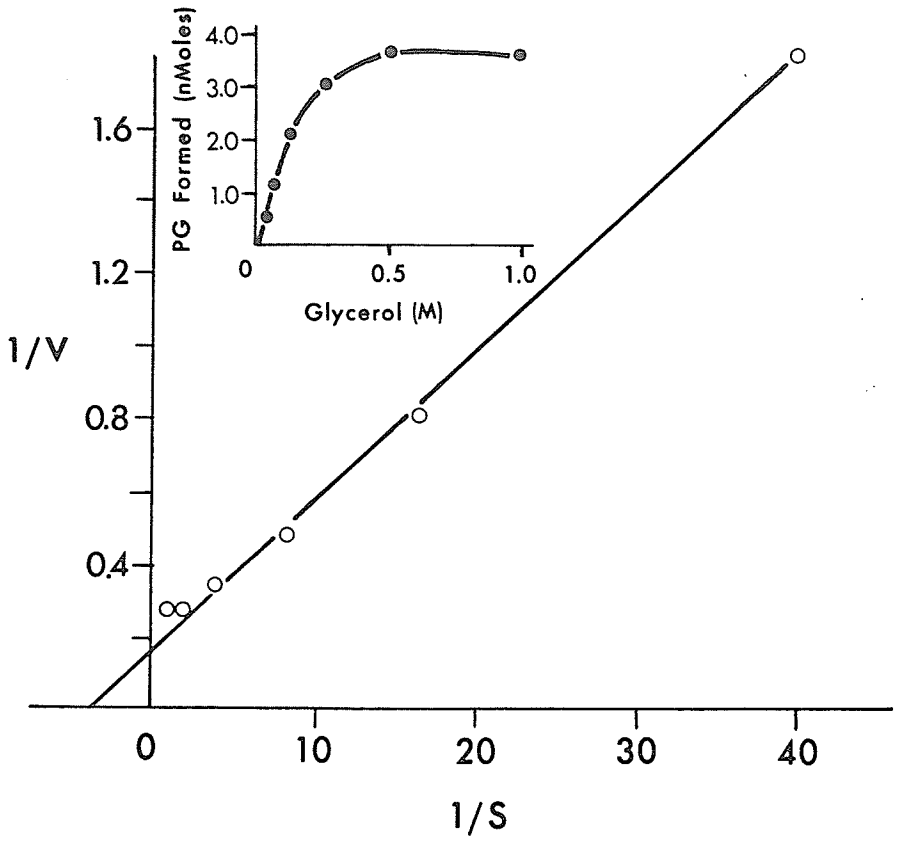


Figure 7. The effect of glycerol concentration on phosphatidylglycerol formation. Assayed as in sec. 3.9.1 in the presence of 16 mM phosphatidylcholine and 8 mM taurodeoxycholate.

forming activities. The conditions for treatment of the enzyme fraction, the radioactive substrate, [U-^{14}C] phosphatidylcholine, and the incubation conditions were identical for both reactions. The only exception to this being the presence of 0.5 M glycerol during the assay for phosphatidylglycerol formation (sec. 3.9.4).

4.1.4.1 Effect of parachloromeruriphenylsulfonic acid (PCMPS)

The effect of 10 min pretreatment of the enzyme containing fraction with various concentrations of PCMPS prior to assay for the two activities was determined (Fig. 8). Identical inhibition curves were observed for both reactions.

4.1.4.2 Effect of heat

Heat stability of the two activities was examined by preincubating the enzyme fraction for various times at 40°C, followed by assaying for the two activities (Fig. 9). It is apparent from these results that the rates of heat inactivation of the two activities are identical.

4.1.4.3 Effect of glycerol concentration

The effects of varying the concentration of glycerol on both phospholipase D activity and on phosphatidylglycerol formation was determined (Fig. 10). An obvious reciprocal relationship was seen to exist between the two activities at all glycerol concentrations. The sum of the radioactivity recovered in the two products was approximately equal at each glycerol concentration.

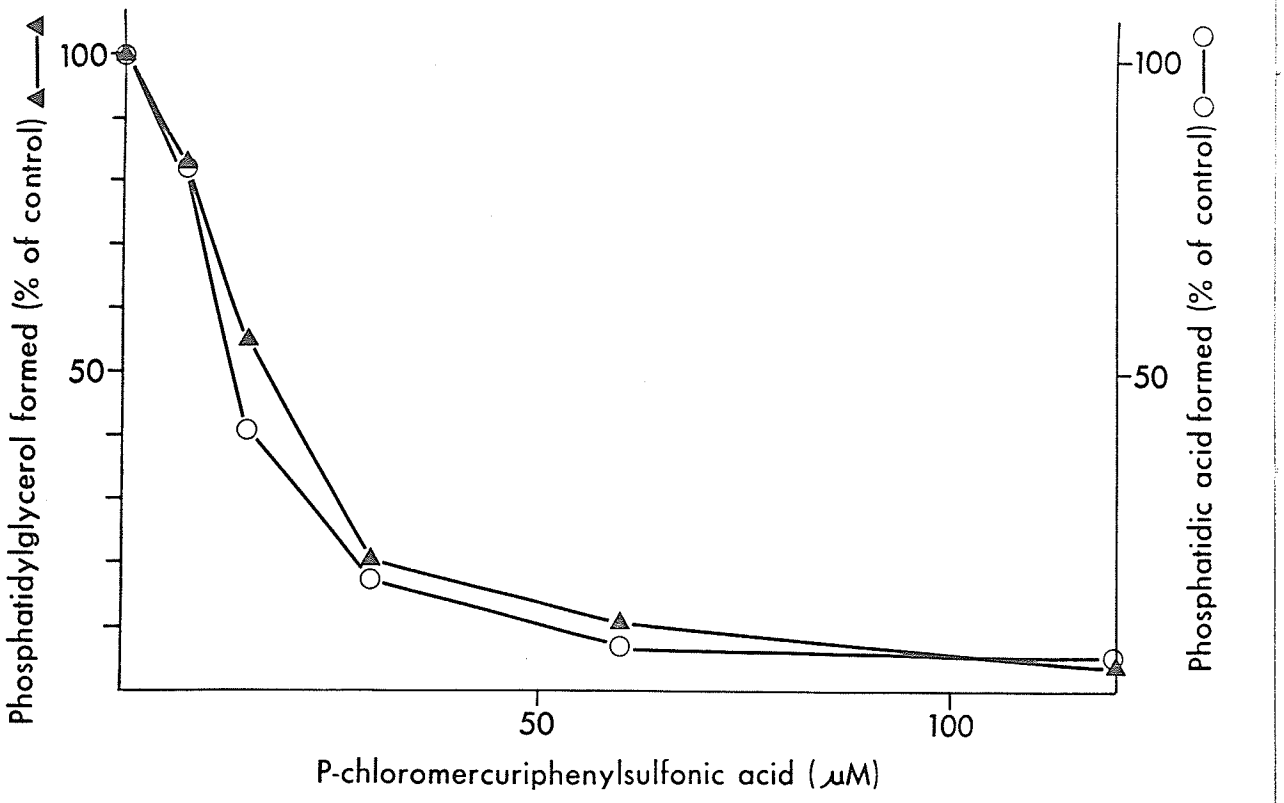


Figure 8. Comparison of the inhibition by PCMPS of phosphatidylglycerol and phosphatidic acid formation from radioactive phosphatidylcholine. The assay was as in sec. 3.9.4.

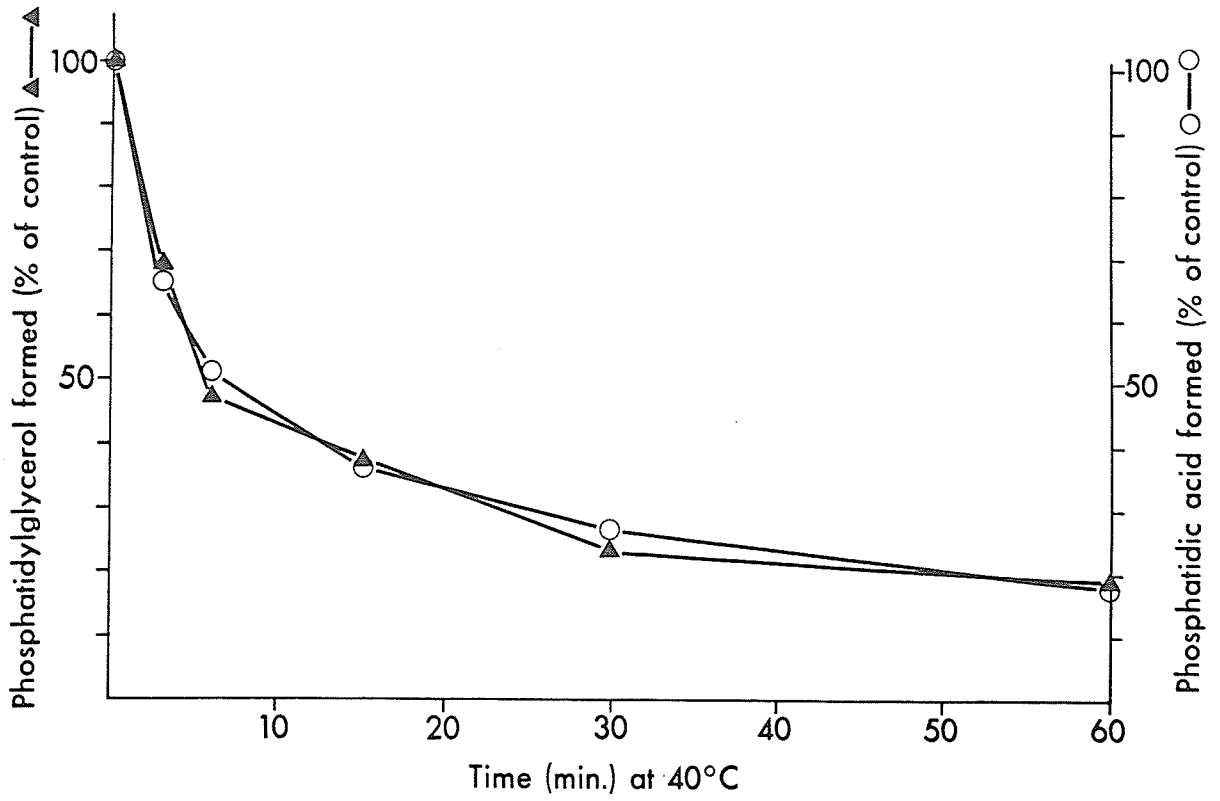


Figure 9. Comparison of the effect of heating the enzyme preparation at 40°C for various times on phosphatidylglycerol and phosphatidic acid formation. The assay was as in sec. 3.9.4 using radioactive phosphatidylcholine as substrate.

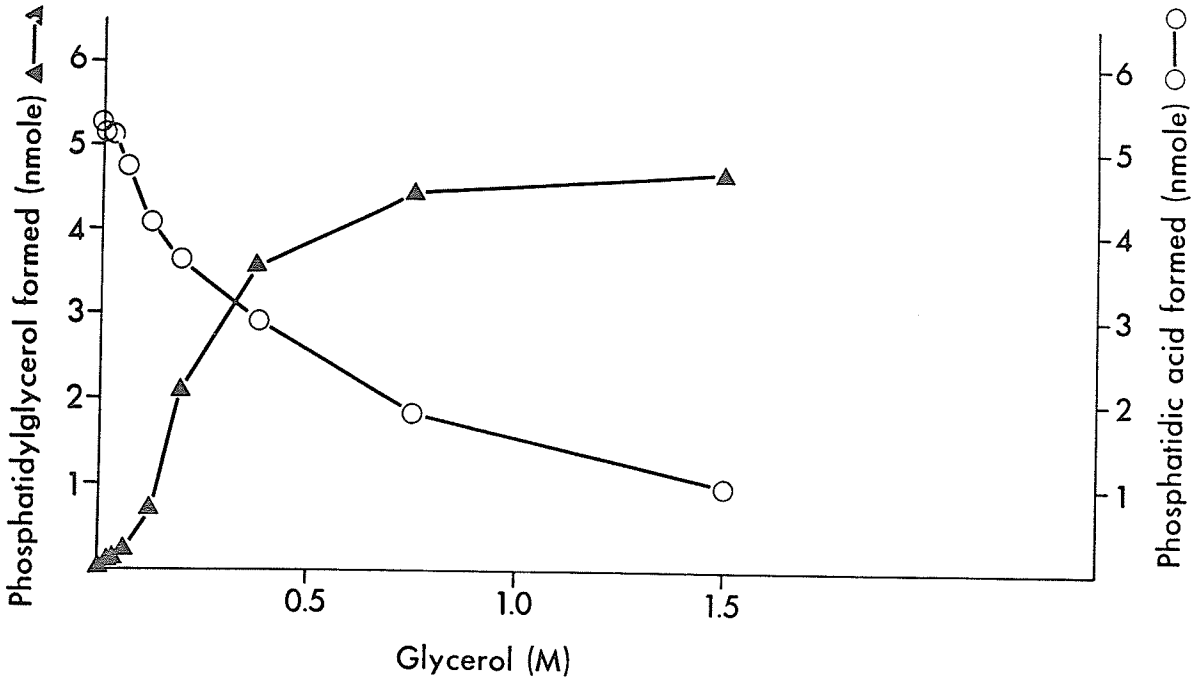


Figure 10. Comparison of the effect of glycerol concentration on the formation of phosphatidylglycerol and phosphatidic acid from radioactive phosphatidylcholine. The assay was as described in sec. 3.9.4.

4.2 Distribution of Phospholipase D in Rat Tissue

The studies of phospholipase D, hydrolytic activity or phosphatidylglycerol forming activity described to this point were carried out with a detergent solubilized preparation of rat brain membranes. The activity was very low in intact membranes by comparison (sec. 4.1.1). The tissue distribution and subcellular location of this enzyme will be useful information in establishing its metabolic function. However, because phospholipase D was only detectable after solubilization, the development of an assay procedure for this enzyme while membrane bound was necessary before carrying out these distribution studies.

4.2.1 Development of assay procedure for membrane bound phospholipase D

The assay procedure was developed using two sources, brain and lung microsomes, in order to establish whether the properties of the enzyme are tissue dependent.

4.2.1.1 The effect of bile salt

The effect of the bile salt taurodeoxycholate (TDOC) on the hydrolytic activity of phospholipase D was examined (Fig. 11c). The enzyme was undetectable in the absence of the detergent. The response of the enzyme to the TDOC was not identical in the two tissues, nevertheless, a 6 mM concentration produced optimal activity in both cases. It is also evident that the appropriate amount of this activator must be present since strong inhibition occurs at high concentrations.

4.2.1.2 Effect of $[^3H]$ phosphatidylcholine concentration

The substrate dependence of phospholipase D (Fig. 11b) was similar in both of these tissue's microsomal fractions. An optimum concentration of 5 mM was observed in both cases above which strong substrate inhibition occured. This effect was less pronounced with the lung enzyme compared to that seen with the brain enzyme.

4.2.1.3 Effect of pH of incubation mixture

The lung and brain enzymes were found to respond identically to the pH of the assay medium (Fig. 11a), with an optimum of pH 6.5.

4.2.1.4 Effect of time, protein and cations

The reaction was linear for 15 minutes at 30°C and up to 200 μg protein per 100 μl assay with both tissues. The addition of 5 mM EDTA to the assay was found to result in 40% stimulation of the activity suggesting that in intact membranes divalent cations are not required. The presence of 5 mM calcium chloride in the assay was found to be slightly inhibitory.

4.2.2 Tissue distribution of phospholipase D

These assay conditions for the hydrolytic activity of phospholipase D in intact membranes were used for the tissue survey of this enzyme. Another assay for the membrane bound enzyme based upon its phosphatidylglycerol forming activity was developed (sec. 4.3) and this was also used for this survey. These are complementary assays in that the hydrolytic reaction depends on saturating concentrations of exogenous

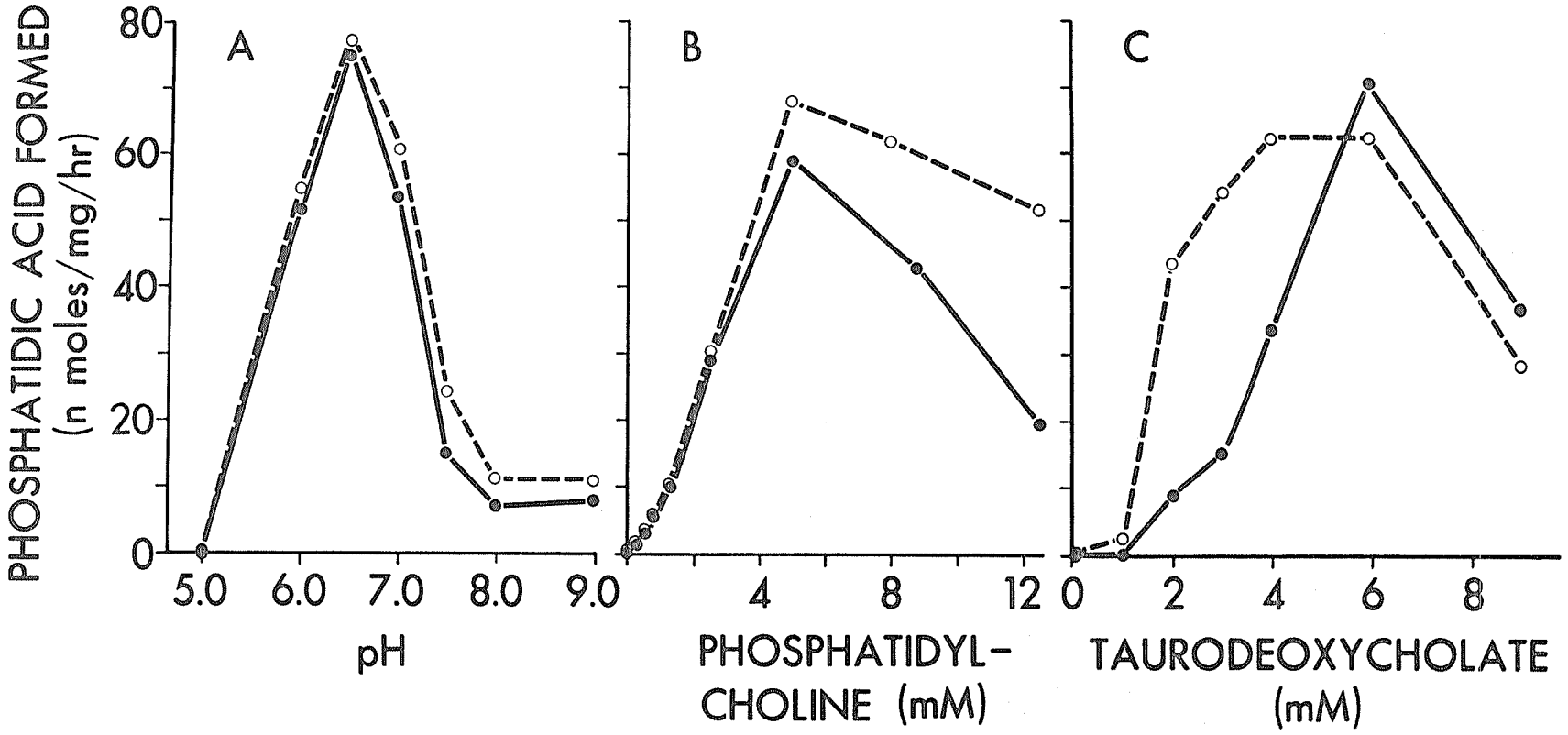
Figure 11. a) The effect of pH on rat brain and lung microsomal phospholipase D. The assay was as described in sec. 3.9.3 with 150 μg of microsomal protein. β,β-dimethylglutarate-NaOH buffer, 50 mM, was used for the pH range, 5.0 to 7.5 glycylglycine-HCl buffer, 50 mM, was used for pH 8.0 and 9.0.

b) The effect of phosphatidylcholine concentration on rat brain and lung phospholipase D. The assay as described in sec. 3.9.3 was used with various amounts of [^{3}H] phosphatidylcholine microdispersion.

c) The dependence of rat brain and lung microsomal phospholipase D on taurodeoxycholate. The assay as described in sec. 3.9.3 was used with the indicated taurodeoxycholate concentrations.

o - - - - o lung

●————● brain


PHOSPHATIDIC ACID FORMED
(n moles/mg/hr)
80
60
40
20
0
A
5.0
6.0
7.0
8.0
9.0
pH
B
4
8
12
PHOSPHATIDYL-
CHOLINE (mM)
C
0
2
4
6
8
TAURODEOXYCHOLATE
(mM)

substrate and TDOC as activator while the phosphatidylglycerol forming assay uses in situ phospholipids as substrate and oleic acid as activator.

Both assay techniques indicated the presence of phospholipase D in every tissue microsomal fraction examined (Tables 7,8), however the amount of enzyme activity varied greatly depending on the tissue examined. Intestinal mucosa and liver displayed much lower activities than did lung, brain or epididymal fat tissue.

4.2.3 Subcellular localization of phospholipase D

The distribution of phospholipase D in the primary subcellular fractions prepared from rat brain and lung tissues (sec. 3.12) was determined using the hydrolytic activity of the enzyme (Table 9a,b). Enrichments of 2.1 and 3.9 fold over the starting homogenates in brain and lung P_3 fractions respectively suggests a microsomal location for phospholipase D. This conclusion is supported by the similar enrichment pattern observed for the microsomal marker enzyme NADPH-cytochrome c reductase and phospholipase D in these subcellular fractions.

4.2.4 Effect of proteases and parachloromercuriphenylsulfonic acid on microsomal phospholipase D activity

Microsomal membrane enzymes have generally been found asymetrically localized on one side of the microsomal membrane (43). Many enzymes require detergents as a result of being enclosed within a substrate impermeable membrane vesicle (43), as with some microsomal (7) and lysosomal (211) enzymes. This is a potential explanation

TABLE 7

Phospholipase D activity of microsomes from various rat tissues

Tissue	Phosphatidic acid formed ($nmol \cdot mg^{-1} \cdot hr^{-1}$)
Brain	46.9
Epididymal fat pad	35.1
Heart	18.8
Intestinal mucosa	0.8
Kidney	18.1
Liver	4.5
Lung	61.6
Skeletal muscle	13.7
Spleen	12.4
Testis	7.8

Microsomes were prepared from 3, 30 day old male Sprague Dawley rats, and 150 μg of each was assayed for PLD as described in sec. 3.9.3.

TABLE 8

Phosphatidylglycerol forming activity of microsomes from various rat tissues

Tissue	Phosphatidylglycerol formed (nmol mg^{-1} hr^{-1})	
	- Oleate	+ Oleate
Brain	0.4	23.8
Epididymal fat pad	0.3	39.2
Heart	0.4	34.1
Intestinal mucosa	0.5	1.3
Kidney	0.3	16.2
Liver	0.4	8.8
Lung	0.6	57.8
Skeletal muscle	0.6	23.2
Spleen	0.9	24.1
Testis	0.1	11.1

Microsomes were prepared from the tissues of 3, 30 day old male Sprague Dawley rats. The incubations were performed in the presence and absence of 4 mM sodium oleate and 100 μg of each microsomal fraction per 50 μl assay.

TABLE 9a

Subcellular localization of phospholipase D in rat brain

	Brain				
Tissue Fraction	Protein mg	Specific activity nmoles/ mg/hr	Enrich-ment	Percent of total activity	Enrich-ment of NADPH-cytochrome C reductase activity
H, Homogenate	720	26.0	1.00	100.0	1.00
P1, Crude nuclei	56	15.6	0.60	4.7	0.58
P2, Crude mitochondria	216	23.1	0.88	26.6	0.73
P2-B, microso-mal wash	48	41.2	1.58	10.5	0.86
P3, microsomes	126	54.8	2.11	36.9	2.06
S, cytosol	132	0.6	0.02	0.4	0.21

Subcellular fraction from brains of 4, 30 day old rats were prepared. Phosphatidic acid formation was measured, sec. 3.9.3, in the presence of 150 µg protein of each fraction.

TABLE 9b

Subcellular localization of phospholipase D in rat lung

	Lung				
Tissue Fraction	Protein mg	Specific activity nmoles/ mg/hr	Enrich-ment	Percent of total activity	Enrich-ment of NADPH-cytochrome C reductase activity
H, Homogenate	561	16.6	1.00	100.0	1.00
P1, Crude nuclei	121	7.6	0.46	9.9	0.53
P2, Crude mitochondria	57	47.0	2.83	28.8	1.20
P2-B, microso-mal wash	13	54.8	3.30	7.6	2.73
P3, microsomes	70	64.3	3.87	48.0	4.34
S, cytosol	152	2.7	0.16	4.4	0.35

Subcellular fraction from lung of 4, 30 day old rats were prepared.

Phosphatidic acid formation was measured, sec. 3.9.3, in the presence of 150 μg protein of each fraction.

for the high degree of activation displayed by phospholipase D in the presence of detergents. The inhibition by proteases and nonpenetrating reagents of a microsomal activity has been widely used to infer that such an activity is located on the outer microsomal membrane surface (137). On the other hand the requirement that deoxycholate (DOC) first disrupts microsomal membranes before the enzyme can be inhibited by these treatments suggests the enzyme is located within the microsomal vesicle.

The results of experiments where microsomes were treated with proteases in the presence and absence of deoxycholate are shown in Table 10. It was found that with intact microsomes (absence of DOC), in which only the outer membrane surface is accessible to protease attack, that 24% (trypsin) or 39% (pronase) of the phospholipase D was destroyed. In disrupted microsomes (presence of 0.05% DOC) which allow proteases access to the inner membrane surface, 52% (trypsin) or 69% (pronase) of the phospholipase D was destroyed.

These results suggest that phospholipase D may exist as two populations of enzymes molecules, one on each side of the microsomal membrane, or possibly as a transmembrane enzyme. Similar experiments employing the nonpenetrating reagent, PCMPS (163) were also carried out (Table 11). About 20% of the phospholipase D activity was inhibited by this reagent in the absence of DOC. With DOC present, allowing PCMPS access to the inner membrane surface, the enzyme

TABLE 10

Effect of proteases on microsomal phospholipase D

Microsomes	Treatment		
	Control	Trypsin	Pronase
Intact	100*	76	61
Disrupted	101	48	31

*Activities are expressed as a percentage of intact microsomes which had a specific activity of 55 $nmol \cdot mg^{-1} \cdot hr^{-1}$. Microsomes were disrupted by the addition of 0.05% deoxycholate. Assays were performed as described in sec. 3.9.3.

TABLE 11

Effect of PCMPS on microsomal phospholipase D

Microsomes	Concentration of PCMPS (μM)			
	0	150	300	600
Intact	100*	90.0	84.5	81.6
Disrupted	110.0	80.7	40.5	9.2

*Activities are expressed as a percent of intact microsomes which had a specific activity of 55 $nmol \cdot mg^{-1} \cdot hr^{-}$. Microsomes were disrupted by the addition of 0.05% deoxycholate. Assays were performed as described in sec. 3.9.3.

was inhibited by 90%. The inhibition of phospholipase D by PCMPS was found to be almost completely reversible by incubation of treated microsomes with the sulfhydryl reducing reagent, dithiothreitol.

4.3 Characteristics of Phospholipase D Activity Towards In Situ Microsomal Membrane Phospholipids

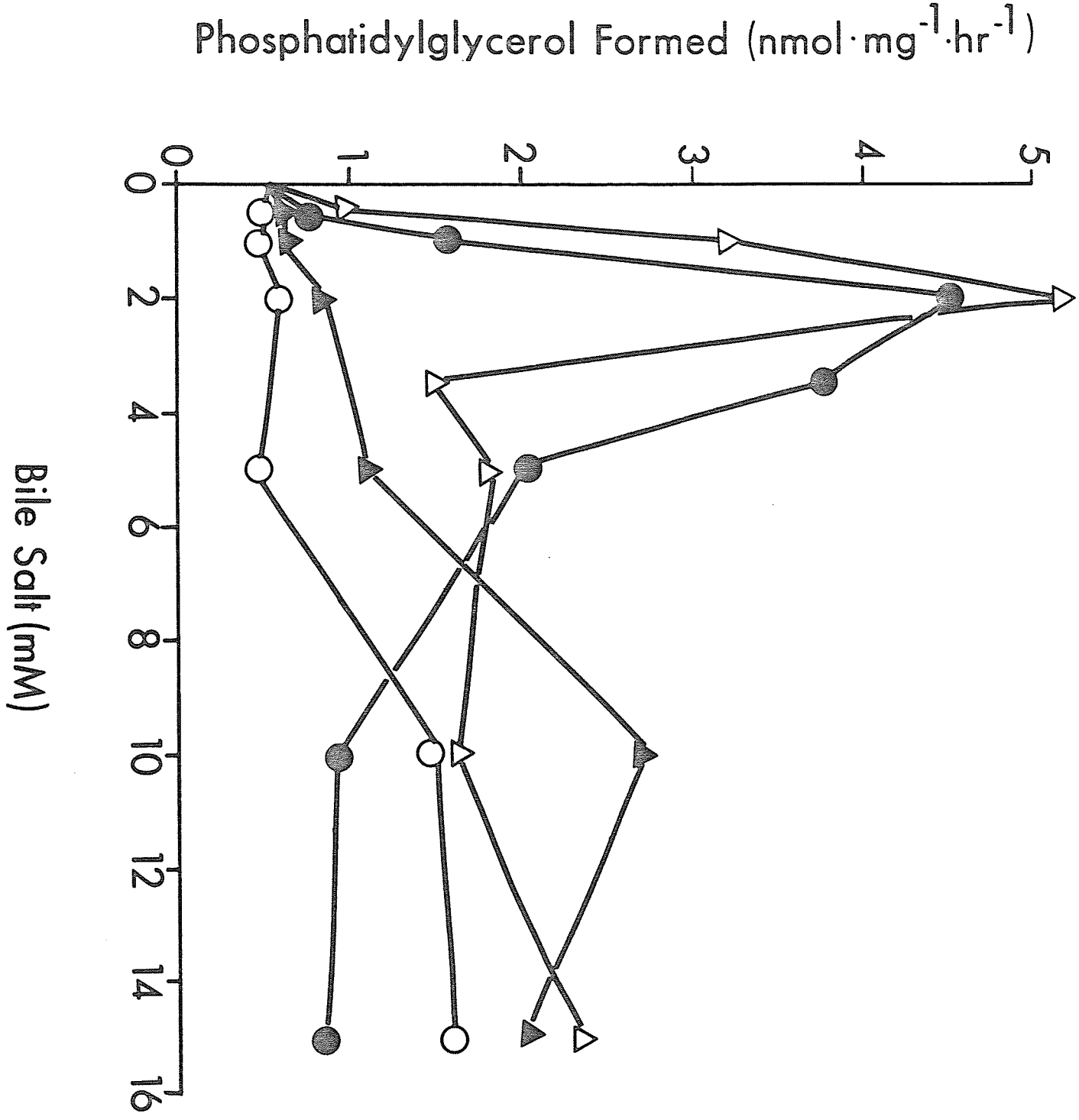
The activity of microsomal phospholipase D was found to be completely dependent upon detergent. (sec. 4.2.1.1). This property of phospholipase D was further investigated as it might provide insights into how this enzyme is regulated within the cell.

An initial question investigated was whether phospholipase D was inactive only toward exogenous phospholipids (sec. 4.2.1.1) or also towards the in situ membrane phospholipids. This was tested by using the phosphatidylglycerol forming reaction of this enzyme to measure its activity towards endogenous membrane lipids. This reaction uses the unlabeled membrane lipids, saturating amounts of $[2-^{3}H]$ glycerol and produces $[^{3}H]$ phosphatidylglycerol which is quantitated by thin layer chromatography as described under methods (sec. 3.9.2).

4.3.1 Effect of bile salts on microsomal phosphatidylglycerol formation

Phosphatidylglycerol formation was measured in the absence and presence of 4 different bile salts. Without the detergents only a slight basal activity was observed of about $0.5\ nmol.mg^{-1}.hr^{-1}$ (Fig. 12). The activity was

Figure 12. Bile salt activation of phosphatidylglycerol formation by rat brain microsomes. Phosphatidylglycerol formation was assayed in the presence of 90 μg of microsomal protein as described in sec. 3.9.2 leaving out the sodium oleate. The bile salt concentrations were varied as indicated ▲, sodium cholate; △, sodium deoxycholate; ○, sodium taurocholate; ●, sodium taurodeoxycholate.


Phosphatidylglycerol Formed (nmol·mg^{-1}·hr^{-1})
0
1
2
3
4
5
Bile Salt (mM)
0
2
4
6
8
10
12
14
16

stimulated by each of the bile salts. It is apparent that the secondary bile salts were more effective than the primary bile salts. This may be related to the differences in the critical micelle concentrations of these detergents which are 2-6 mM for deoxycholate and taurodeoxycholate and 10-14 mM for cholate and taurocholate (79). This clearly indicates that rat brain phospholipase D requires detergent to act effectively on the phospholipids surrounding it in the microsomal membrane, as well as requiring detergents to act on exogenous substrates. The protease and PCMPS treatments indicated that a portion of this enzyme is located on the outer side of the microsomal membrane, therefore compartmentation of phospholipase D within the microsomal vesicle should not be an explanation for its latency. Furthermore, glycerol is known to freely cross microsomal membranes (139), and so should be accessible to an enzyme at either membrane surface. Further experiments were carried out to provide evidence that glycerol is available to phospholipase D on both sides of the microsomal membrane.

4.3.2 Effect of preincubating microsomes with [^{3}H] glycerol

Experiments allowing glycerol longer times to cross the microsomal membrane, and so equilibrate in the lumen of these vesicles were performed. Microsomes and [^{3}H] glycerol were preincubated for 0, 1 and 2 hr at 0^{o}C before assaying. After this treatment the same detergent

dependence was observed. This indicates that the rate of glycerol diffusion into the microsomes is not the limiting factor nor the cause of the observed latency.

4.3.3 Effect of amphiphilic rat brain lipids on microsomal phospholipase D

Bile salts are considered physiological activators of certain intestinal enzymes (134), however, absence of these steroids in brain tissue suggests they may be mimicing some other amphiphile when they activate phospholipase D of rat brain. Experiments were therefore carried out testing a variety of lipids which occur in brain tissue for their ability to activate phospholipase D. As seen in Table 12 sodium oleate was found to be the only one of these lipids capable of activating the enzyme significantly. Oleate was found to be 10 fold more effective an activator than deoxycholate under these conditions.

4.3.4 Latency and fatty acid activation of PLD from various rat tissues

Microsomal fractions prepared for a range of rat tissues were assayed for phosphatidylglycerol formation in the absence and presence of oleic acid (Table 8). It was found that in each tissue the enzyme activity was very low in the absence of an activator. Oleic acid in each case was found to stimulate phospholipase D activity.

4.3.5 Effect of fatty acids on phospholipase D

Additional studies were undertaken to determine the details of fatty acid activation of microsomal phospholipase D.

TABLE 12

Effect of some potential activators on microsomal phospholipase D

Substance	Concentrations tested (mM)	Maximum phosphatidylglycerol formation $nmol \cdot mg^{-1} \cdot hr^{-1}$
No addition		1.0
Oleic acid	4	56.8
Cardiolipin	1,4	1.6
Phosphatidic acid	1,4	2.4
Phosphatidylglycerol	1,4	1.8
Phosphatidylinositol	1,4	1.2
Phosphatidylserine	1,4	1.4
Lysophosphatidylcholine	1,4,10	1.0
Lysophosphatidylethanolamine	1,4,10	1.8
Mixed gangliosides	1,4,10	1.4
Palmitoyl coenzyme A	1,4,10	0.4
Oleoyl coenzyme A	4	0.2
Triton X-100	1,4,10	2.0
SDS	1,4,10	1.2
Cetyltrimethyl ammonium bromide	1,3,6	1.0
Miranol H2M	1,3,6	1.0
Octyl-β-D-glucopyranoside	1,3,6	0.8

The substances were added as solvent solutions, dried under nitrogen flow, then resuspended in buffer. The incubations contained 100 μg of microsomal protein per 50 μl assay.

4.3.5.1 Fatty acid specificity for activation of phospholipase D

Oleate and palmitoleate were found to be the most effective of a variety of fatty acids sodium salts tested as potential activators (Table 13). All of the unsaturated species activated to some degree in contrast to the saturated ones, which with the exception of lauric acid (C-12), were ineffective. Methyl oleate and oleic alcohol were ineffective while oleylamine was 25% as effective as oleate.

4.3.5.2 Relationship between microsomal membrane concentration and the oleate required for activation of phospholipase D

The concentration of oleate required for activation of phospholipase D depended upon the amount of microsomal protein present in the incubation mixture (Fig. 13). Optimal activation occurred at an oleate concentration of 2 μmol/mg microsomal protein.

4.3.5.3 Relationship between exogenous phosphatidylcholine concentration and the oleate required for phospholipase D

The ability of oleate to stimulate the activity of phospholipase D toward exogenous phospholipid substrate was tested using a $[^3H]$ phosphatidylcholine microdispersion, and measuring the production of phosphatidic acid (Fig. 14). At a constant microsomal membrane concentration, the optimal oleate concentration was found to depend directly on the amount of substrate lipid in the incubation. It was

TABLE 13

Effect of fatty acids on the phosphatidylglycerol forming activity of phospholipase D

Fatty Acid (Sodium Salt)	Phosphatidylglycerol formation $nmol \cdot mg^{-1} \cdot hr^{-1}$	% *
None	0.28	0.6
Butyrate	0.48	1.0
Caprylate	0.24	0.5
Laurate	22.78	47.3
Palmitate	0.48	1.0
Palmitoleate	49.26	102.4
Stearate	0.18	0.4
Oleate	48.10	100.0
Linoleate	34.12	70.9
Linoleneate	30.42	63.2
Arachidonate	26.82	55.7
Methyl oleate	0.38	0.8
Oleic alcohol	1.20	2.5
Oleoyl amine	11.54	24.0

*These values are compared to oleate which is 100%. Fatty acid sodium salts were added as aqueous solutions to give a 4 mM concentration. The incubations contained 100 µg of microsomal protein per 50 µl. Assays were performed as described in sec. 3.9.2.

Figure 13. Effect of various microsomal membrane protein concentrations on the activation of phospholipase D by oleate. Phosphatidylglycerol formation was determined as described in sec. 3.9.2 with different amounts of microsomal membrane. Δ , 25 μg; ● , 50 μg; ▲ , 100 μg; ○ , 200 μg. The oleate concentration was varied as indicated.

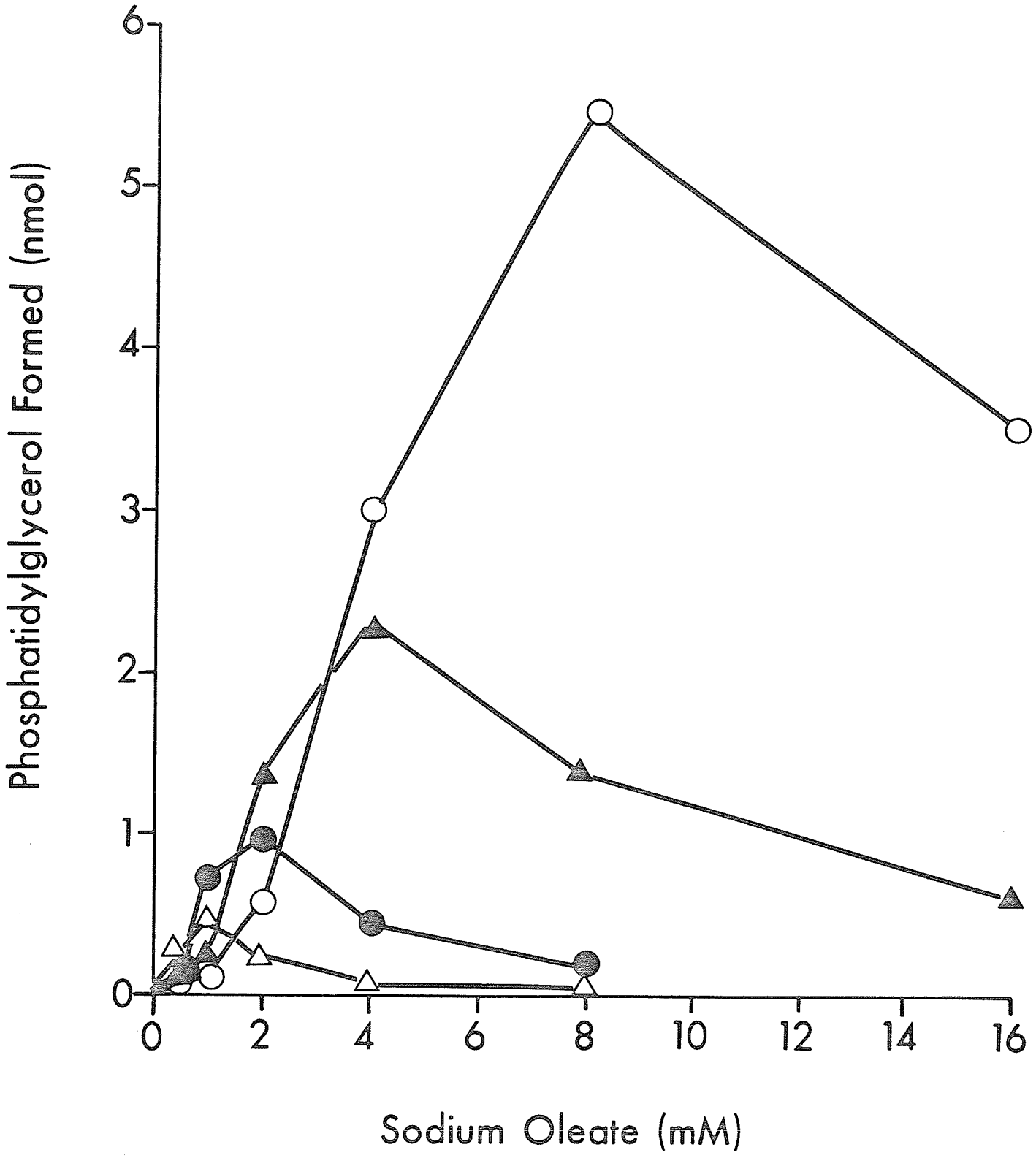
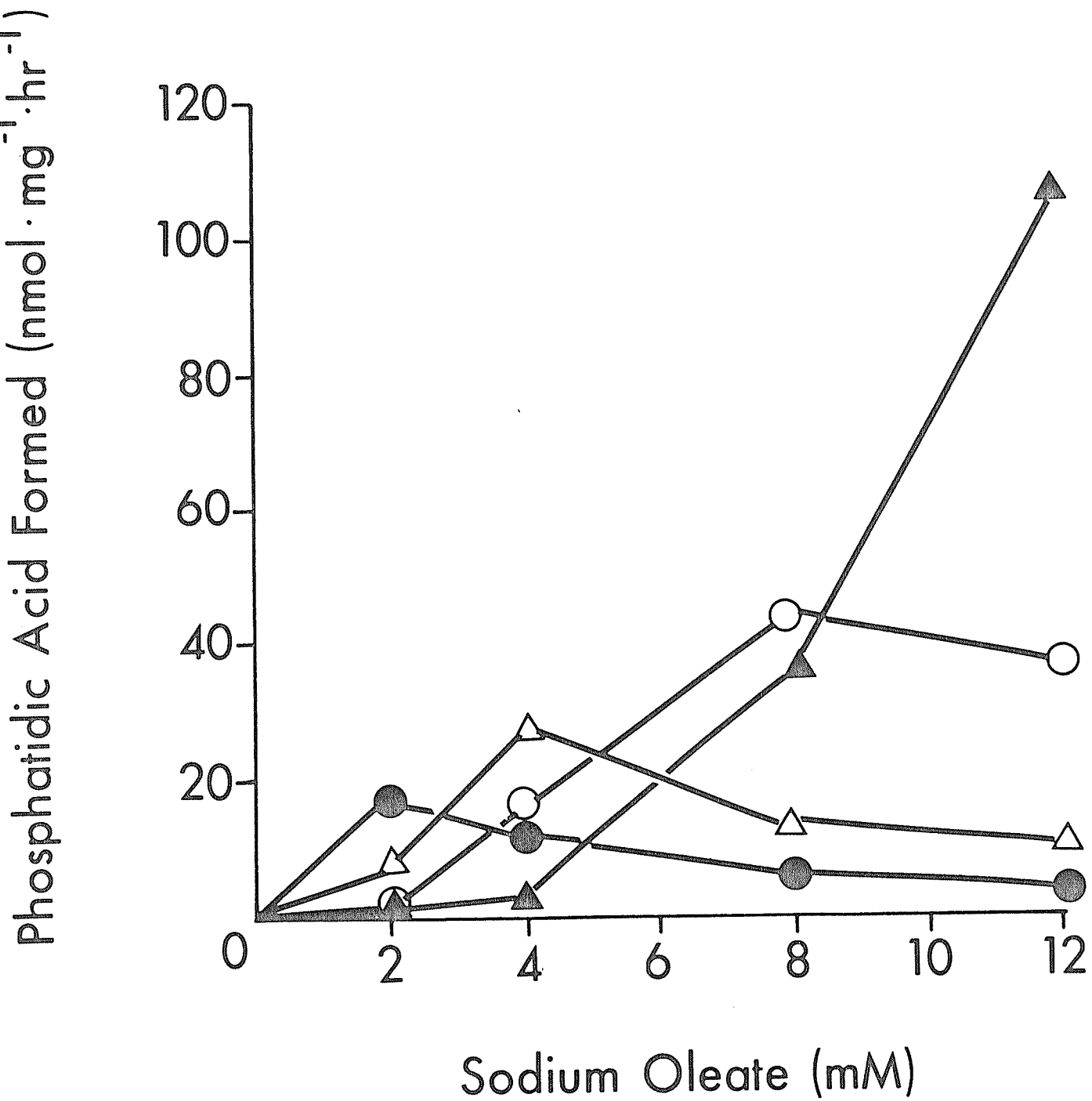

Phosphatidylglycerol Formed (nmol)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
Sodium Oleate (mM)

Figure 14. The effect of varying phosphatidylcholine concentration on the oleate activation of phospholipase D. Phosphatidic acid formation was determined as in sec. 3.9.3 in the presence of 150 μg of microsomal protein. The indicated concentration of [^{3}H] phosphatidylcholine was added. ● , 1 mM; Δ , 2 mM; o , 4 mM; ▲, 8 mM.


Phosphatidic Acid Formed (nmol · mg⁻¹ · hr⁻¹)
120
100
80
60
40
20
0
2
4
6
8
10
12
Sodium Oleate (mM)

maximal at an oleate to phosphatidylcholine molar ratio of 2:1.

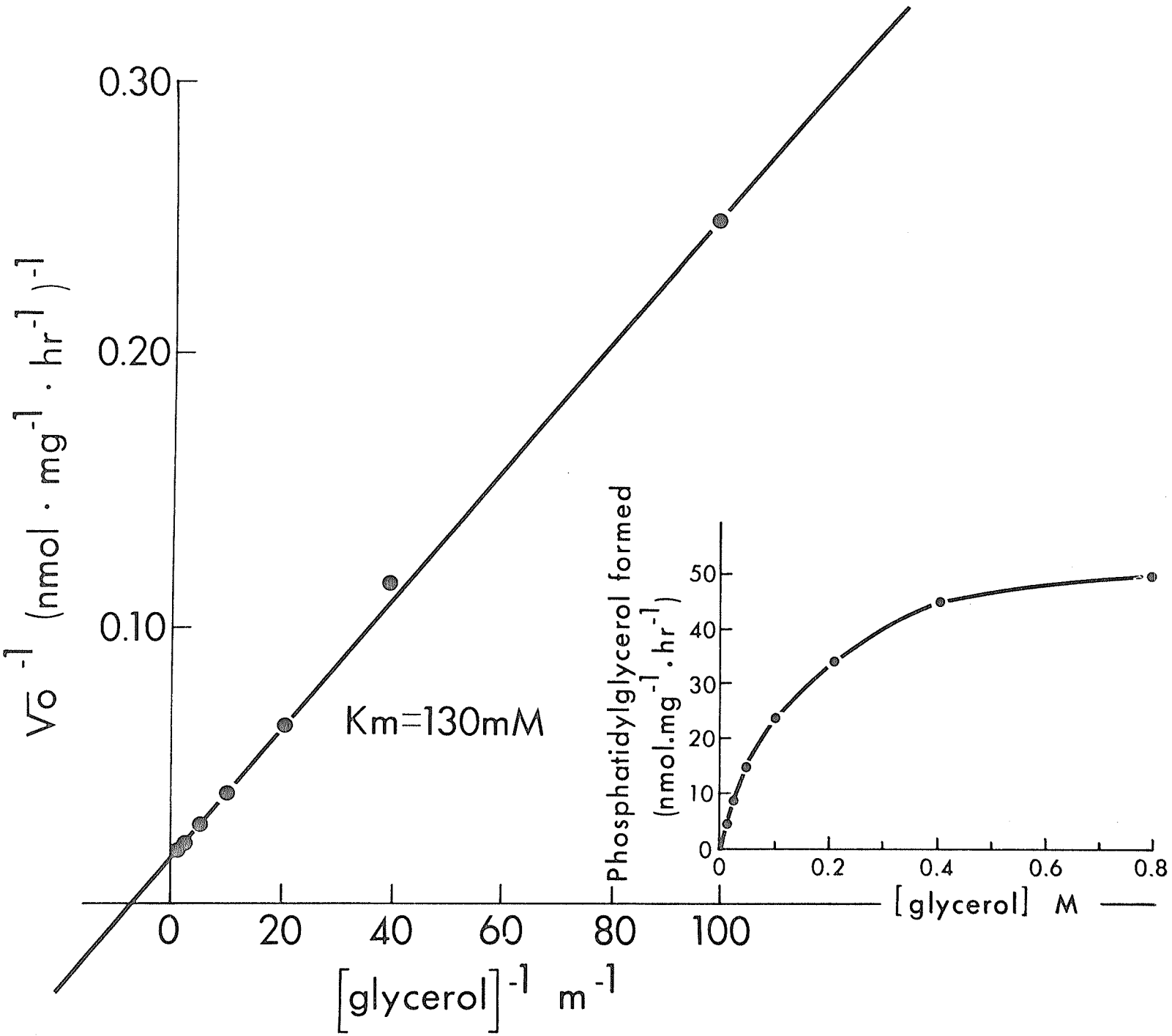
4.3.5.4 The effect of oleate and the binding of phospholipase D to microsomal membranes

The possibility that activation of phospholipase D results from its being solubilized from the microsomal membrane was examined. Concentrations of oleate which cause optimal stimulation of phospholipase D were mixed with microsomes and allowed to remain at 0°C for 10 min. Following this treatment the microsomes were pelleted by centrifugation at 100,000 x g for 60 min. Phospholipase D was assayed in the microsomes after this treatment and 80% of its activity was found to have remained associated with them.

4.3.5.5 pH optimum and apparent K_m for glycerol of fatty acid activated phospholipase D

The optimal pH for phosphatidylglycerol formation by oleate activated phospholipase D was found to be 6.5 which was unchanged from the optimum seen for PLD either in solubilized state (Fig. 4) or when microsomal bound, but activated by TDOC (Fig. 11a). The apparent K_m for glycerol under these conditions of oleate activation was determined to be 130 mM (Fig. 15). This was not greatly different from the K_m observed for the solubilized, partially purified form of the enzyme (Fig. 7).

Figure 15. The effect of glycerol concentration on the oleate activated phospholipase D catalysed phosphatidylglycerol formation. Phosphatidylglycerol formation was determined using 100 μg of microsomal protein as in sec. 3.9.2 at the indicated glycerol concentrations.


0.30
0.20
0.10
V_o^{-1} (nmol · mg^{-1} · hr^{-1})$^{-1}$
Km=130mM
0
20
40
60
80
100
[glycerol]$^{-1}$ m^{-1}
Phosphatidylglycerol formed (nmol.mg^{-1}.hr^{-1})
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
[glycerol] M

4.3.5.6 The effect of taurodeoxycholate on oleate activated phospholipase D

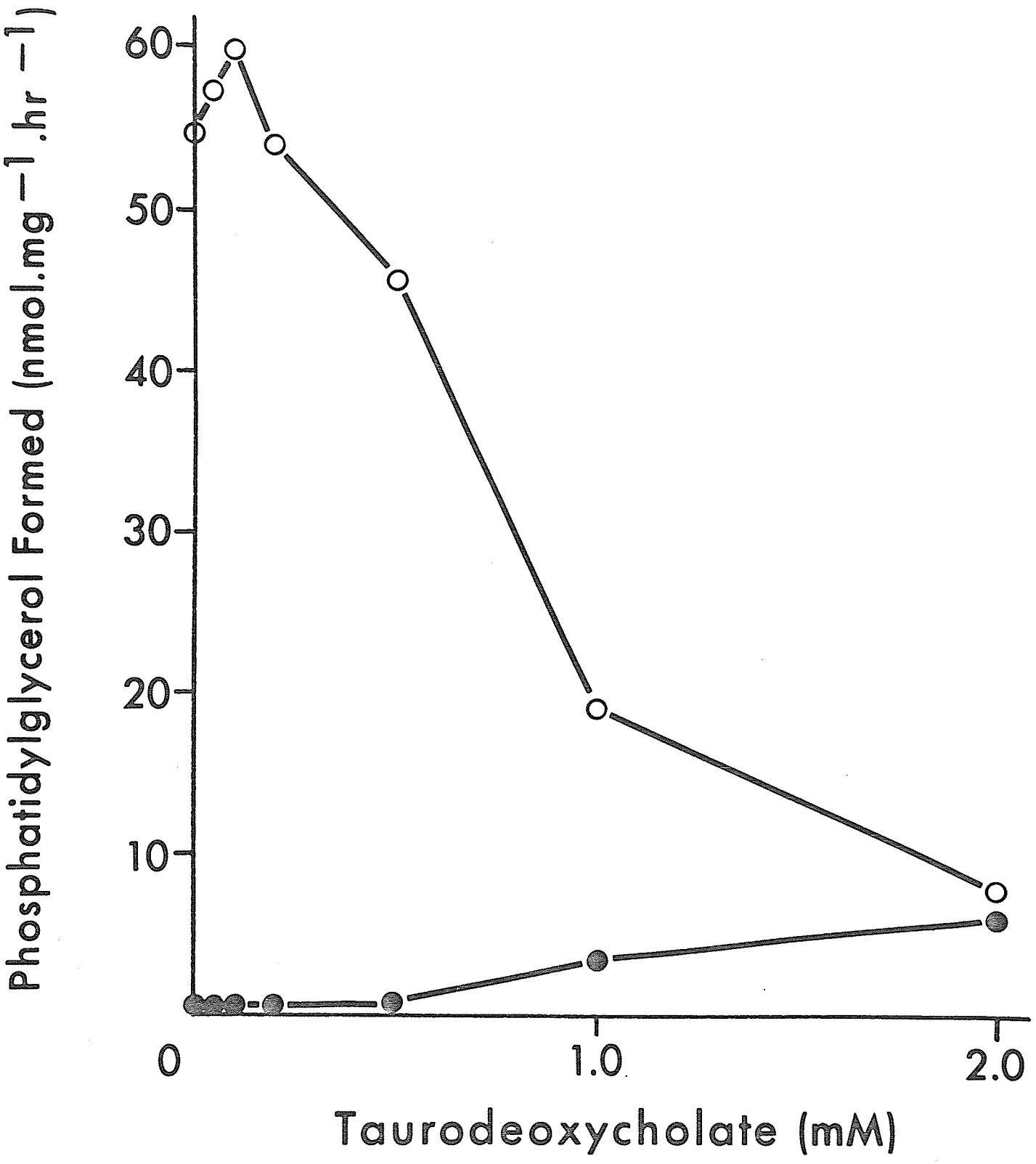
Only the bile acids and fatty acids were effective activators of phospholipase D. Experiments were carried out to determine if these two activators were acting by the same mechanism. The effects of the two compounds should be additive if they act by independent mechanisms. It is seen that the enzyme, maximally activated by oleate could be further stimulated slightly by low concentrations of the bile salt (Fig. 16). An inhibition was seen at higher concentrations with 80% loss of activity at 2 mM of the bile salt. The slight additive effective of TDOC observed, took place at a much lower concentration than seen for this bile salt alone (Fig. 12). No additive activation was seen at that previous optimal TDOC concentration suggesting that bile salt and oleate activate by the same mechanism.

4.3.5.7 Influence of temperature on the rate of oleate activated phospholipase D

The influence of the lipid environment on the activity of membrane enzymes is often reflected in a discontinuous Arrhenius plot (171). These "break points" correlate with changes in the state of the lipids from a gel like to fluid state, releasing constraints on certain enzymes, and observed as a lowered activation energy (119).

The possibility that phospholipase D is also influenced by the physical state of the membrane lipids was investigated by determining the effect of temperature on its activity.

Figure 16. The effect of various taurodeoxycholate concentrations on the oleate activated phospholipase D. Phosphatidylglycerol formation was determined as in sec. 3.9.2 at various sodium taurodeoxycholate concentrations in the presence, o, or absence, ● , of 4 mM sodium oleate with 100 μg of brain microsomes.


Phosphatidylglycerol Formed (nmol.mg^{-1}.hr^{-1})
60
50
40
30
20
10
0
1.0
2.0
Taurodeoxycholate (mM)

In the absence of oleate, the enzyme was highly latent even when assayed at 37, 40, 45 or 50°C. In the presence of detergent, the activity was optimal at 30°C. A striking sensitivity to heat was observed at higher temperatures (Fig. 17). An Arrhenius plot of these data revealed a break point in this plot between 15 and 20°C, suggesting that this activity was affected by the fluidity of surrounding membrane lipids (Fig. 18).

Figure 17. Phospholipase D activity assayed at various incubation temperatures. Phosphatidylglycerol formation was determined in the presence of 100 μg of microsomal protein as in sec. 3.9.2. The incubation temperature was varied as indicated.

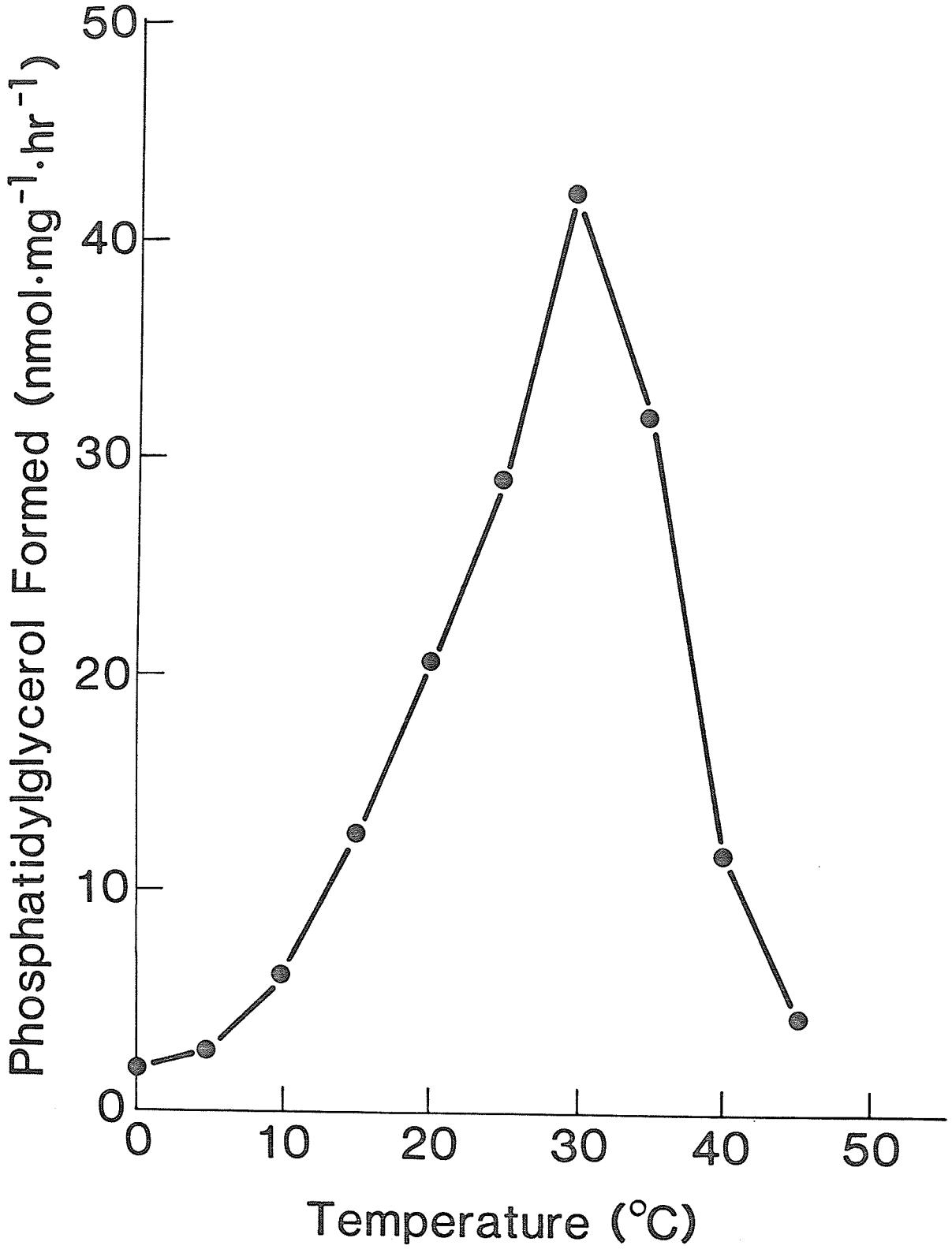
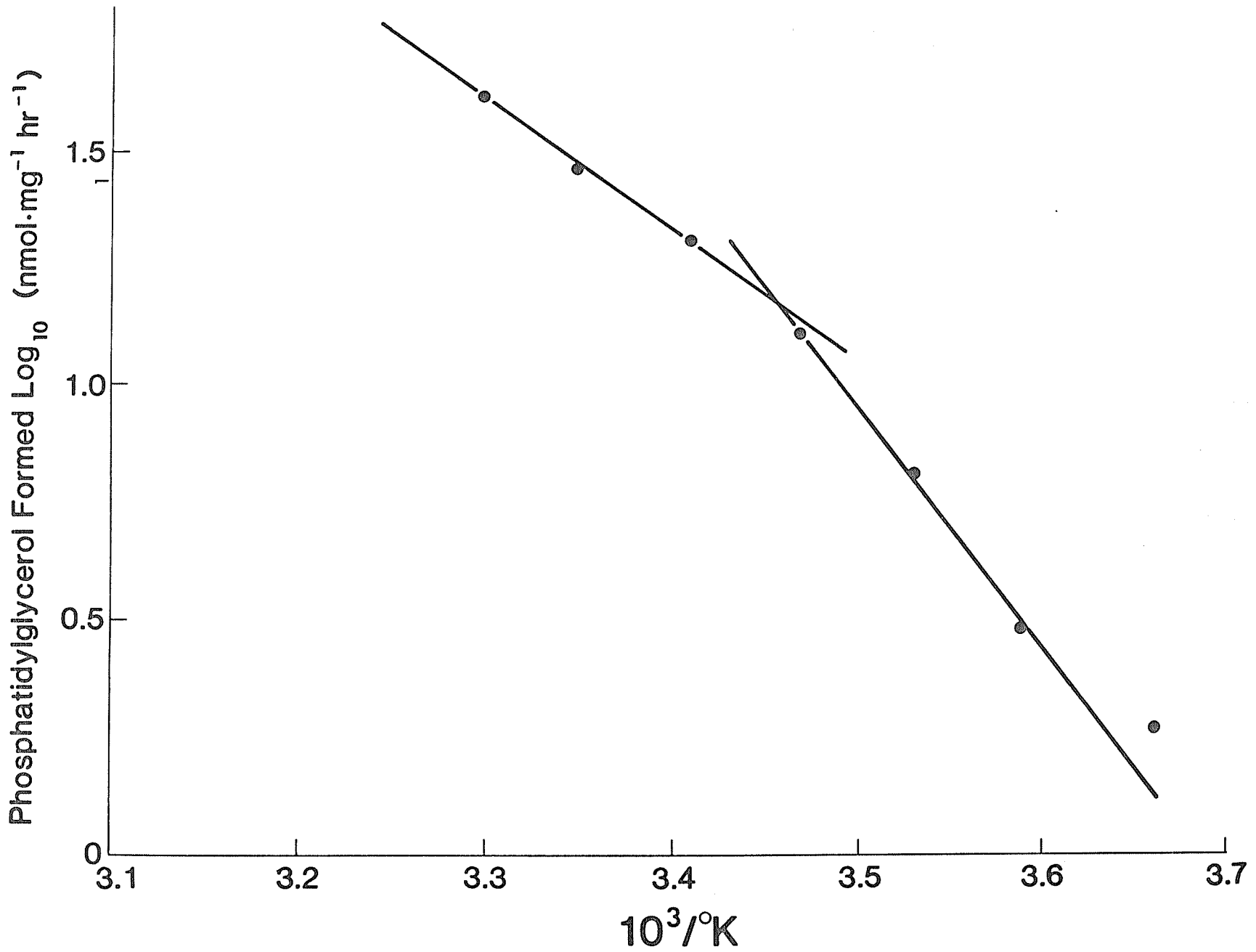

Phosphatidylglycerol Formed (nmol·mg⁻¹·hr⁻¹)
50
40
30
20
10
0
0
10
20
30
40
50
Temperature (°C)

Figure 18. Arrhenius plot of phospholipase D activity measured at various temperatures. The data from 0 - 30°C from Fig. 17 were replotted.


Phosphatidylglycerol Formed Log10 (nmol·mg-1 hr-1)
1.5
1.0
0.5
0
3.1
3.2
3.3
3.4
3.5
3.6
3.7
10³/°K

5. DISCUSSION

This study has been directed at characterizing certain enzymatic properties of phospholipase D (PLD) from a mammalian source. The work has dealt with three aspects of this activity in rat tissues. The first aspect concerns the ability of PLD to catalyse a phospholipid polar head group exchange reaction, in addition to its more usual head group hydrolytic activity. The second aspect dealt with an examination of the properties of PLD in intact tissue extracts. Previous reports about PLD from mammalian tissues (169,195) have dealt with studies of the enzyme in a detergent solubilized state. The final aspect of PLD investigated attempted to understand the reasons for the detergent requirement observed for this enzyme. Unsaturated free fatty acids are found to be the most effective activators of PLD and their activation of the enzyme was investigated.

5.1 Characteristics of a Phosphatidylglycerol (PG) Forming Activity of Rat Tissue

The initial observations leading to these studies were made during the purification of rat brain PLD. Sepharose 4B column fractions of detergent extracts from rat brain, containing 20% glycerol as an enzyme stabilizer, were assayed for PLD with $\left[U\text{-}^{14}C\right]$ phosphatidylcholine as labeled substrate. Phosphatidic acid (PA) formation was measured by extraction of lipids from these incubations followed by their separation using two dimensional thin layer chromatography. In addition to the expected product PA, another radioactive lipid was

produced in significant quantities. The mobility of this lipid was found to be similar to that of phosphatidylglycerol (PG) when it was rechromatographed with a range of standard phospholipids. This observation suggested that the fraction derived from rat brain contained an enzyme system capable of synthesizing PG. Before proceeding further in characterizing the enzyme(s) involved an unequivocal product identification was required.

The structure of this lipid was clearly established to be phosphatidylglycerol (PG), based upon a range of analytical techniques.

The thin layer chromatographic systems used for its identification are known to result in different relative mobilities and separations of phospholipids (164,148,73). Co-chromatography of the lipid with standard PG therefore suggests that it is the same compound. The water soluble products remaining after deacylation of phospholipids by alkaline treatment are unique glycerol phosphoryl esters which can be resolved from each other in the paper chromatographic systems used in these studies (37). The co-chromatography of the water soluble products from the labeled lipid and from standard PG suggests that the water soluble backbone of these two compounds is identical and consists of glycerol phosphoryl glycerol (37). Acetic acid hydrolysis of the phosphodiester bonds of phosphoglycerides is only possible with those species possessing a free hydroxyl group vicinal to the phosphate moiety (29). Phospholipids which possess this structure and which have been shown to react in

this way are phosphatidylinositol (75), cardiolipin (125) and PG (226). The lipid was found to react under these conditions to yield a water soluble product which co-chromatographed with α-glycerol phosphate, the product expected only from PG (226). This indicates that the lipid contains glycerol esterified to phosphate with at least one free hydroxyl group vicinal to the phosphoester bond. The possibility of glycerol being esterified at carbon 2 is unlikely since the β-glycerol phosphate that would result from this hydrolysis is known to be separated from α-glycerol phosphate in the chromatographic system used (98). Additional evidence for the location of the phosphoester bond at an α-carbon of the glycerol and also for the presence of two free hydroxyl groups is provided by the ability of the compound to form an isopropylidene derivative. The formation of this derivative requires adjacent hydroxyls since only the five membered ring cyclic ketal is stable (152).

The ability of phospholipases C and D to degrade the labeled lipid and in each case to yield the product expected from PG provides additional evidence indicating that it is phosphatidylglycerol.

This structural study of the labeled lipid confirmed that the detergent extract of rat brain membranes contained an enzymatic mechanism for the production of PG from phosphatidylcholine. The nature of this enzyme(s) was therefore investigated.

The de novo pathway for PG synthesis is the only known route for the production of this phosphoglyceride in mammalian

tissues (10). This pathway was first reported by Kiyasu et al. (111) using a rat liver preparation, and has been subsequently shown to occur analogously in all organisms examined (198). Two separate reactions are required for PG production by this route;

1. CDP-diglyceride + sn-glycerol-3-phosphate ⟶ phosphatidylglycerol phosphate + CMP
2. phosphatidylglycerol phosphate + H_2O ⟶ phosphatidylglycerol + Pi

In the first reaction sn-glycerol-3-phosphate and CDP-diglyceride are substrates for the formation of phosphatidyl-glycerolphosphate catalysed by the enzyme CDP-diglyceride: sn-glycerol-3-phosphate phosphatidyltransferase. This compound is then dephosphorylated by phosphatidylglycerolphos-phate phosphatase. This pathway for PG synthesis has been demonstrated to occur in rat (151) and sheep brain (190). Studies of the subcellular distribution of this enzyme sequence have demonstrated it to be associated principally with the mitochondrial fraction of liver (111) and brain (151).

The properties of the enzyme system producing PG described in this thesis suggest it is distinct from this de novo pathway. A major point of difference is in substrate requirement. While the de novo route utilizes sn-glycerol-3-phosphate and CDP-diglyceride, the enzyme system examined here requires only glycerol and phosphatidylcholine. With [U-^{14}C] phosphatidylcholine as substrate, PG formation was not observed unless glycerol was present in the incubation (Fig. 10). The same reaction characteristics were seen with

unlabeled phosphatidylcholine and [2-^{3}H] glycerol (Fig. 7).

An alternative mechanism for PG formation consists of a direct exchange of free glycerol for the polar head group of phosphoglycerides. This type of reaction has been described for the formation of phosphatidylcholine (44), phosphatidylethanolamine (18), phosphatidylserine (90), and phosphatidylinositol (143). In bacteria, cardiolipin has been shown to be produced by an exchange reaction involving two phosphatidylglycerols (86). Although an exchange mechanism for the production of PG had not been previously reported for animal tissues, such a finding might not be surprising given the existence of exchange activities for the synthesis of the other phosphoglycerides.

Besides the possibility of the existence a novel glycerol phospholipid head group exchange enzyme, other possible explanation for this observed PG synthesis are available. Potentially, one of the four mammalian head group exchange enzymes referred to above could be responsible. Protein fractions specific for choline, ethanolamine and serine have been prepared from brain (132),(197), however it is possible that at the high glycerol concentrations required for PG production, this alcohol may also be transferred by any of these enzymes. Another enzyme which may be responsible for this observed PG production is the PLD present in these tissues (169). PLD from plants is known to catalyse the exchange of alcohols with the polar head groups of phospholipids (226) (34) (200).

There are distinct differences that distinguish

the PG forming activity described here from the previously described head group exchange enzymes of rat brain. The base exchange enzymes for choline, ethanolamine and serine exchange are known to require calcium ion for activity. In intact microsomes the optimum calcium concentration was 0.25 mM, 0.8 mM and 1.0 mM for choline, ethanolamine and serine exchange respectively, while in the detergent solubilized state the optimum concentrations were higher, 8 mM for choline and ethanolamine and 25 mM for serine (168). The PG forming activity observed in this study was not dependent on calcium or other divalent cations since it was fully active in the presence of 50 mM EDTA or EGTA. The addition of a variety of cations to the incubation mixture was not observed to result in the stimulation of the activity, (Fig. 5) but rather caused some degree of inhibition in each case. The base exchange activities are optimal at alkaline pH values, in contrast to PG formation which was observed to be most active at pH 6-6.5 (Fig. 4). In intact microsomes the optimal pH was 9.0 for all three exchange activities while in detergent solubilized microsomes this was shifted to 7.2 (168).

The criteria of cation requirement and pH optimum also distinguish the inositol exchange enzyme from this PG forming activity. Inositol exchange requires manganese ion for activity (143,193), while this cation inhibited PG formation by about 50% at 10 mM. The inositol exchange is optimal at about pH 7.4 (192) slightly more alkaline then the pH optimum of 6.5 observed for PG formation. Exchange

of inositol appears to be a reaction between the inositol moiety of phosphatidylinositol and free inositol (192,14). With the liver inositol exchange activity phosphatidylcholine and phosphatidylethanolamine were not acceptors for the exchange (192). This substrate preference, observed for inositol exchange, is quite different from that seen for PG formation in this study (Table 6). PG formation occured preferentially with phosphatidylcholine as substrate, and not with phosphatidylinositol.

The differences in properties of the polar head group exchange enzymes for choline, ethanolamine, serine and inositol distinguish these activities from the PG forming enzyme reported here. There does however seem to be similarity between this PG forming activity and rat brain PLD recently reported (169). This hydrolytic activity is extracted from lyophilized rat brain homogenate by the same procedure as used in this study for PG forming enzyme (195). In this solubilized state, neither of these activities are dependent on divalent cations. The addition of 12.5 mM EDTA was without effect upon the hydrolytic reaction (169), however, unlike the PG forming activity PLD was stimulated by about 50% by 5 mM calcium chloride (169). It is possible however that this effect of calcium may not be on PLD itself but rather on an interfering activity, phosphatidic acid phosphatase (PAP) (198). This activity from brain (72) and other tissues (228) is inhibited by calcium and other divalent cations. Therefore during the assay for PA formation, PAP may lower the apparent activity of PLD by

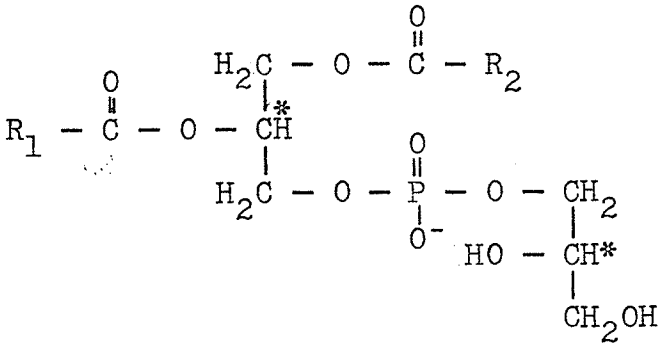
consuming PA as it is produced. When calcium is added, the inhibition of PAP is observed as an increase in PA accummulation, and an apparent stimulation of PLD. The formation of PG, if by PLD, would not however be stimulated since it is not a substrate of PAP.

These similarities in cation requirements and pH optima between PLD and the PG forming activity, prompted a closer comparison of their properties in an attempt to establish their possible identity. Rat brain PLD is very sensitive to the sulfhydryl directed reagent, parachloromercuriphenylsulfonic acid (PCMPS) which at 60 μM inhibits its activity by greater then 90% (169). The sensitivity of both PLD and PG forming enzymes to this reagent was compared and found to be very similar (Fig. 8). PLD has been shown to be relatively heat labile (195). When the fraction containing PLD and the PG forming activity was heated for various times at 40^{o}C, these were both inactivated in an identical fashion (Fig. 9).

The effect of glycerol concentration on the two reactions was examined (Fig. 10). An almost stoichiometric reciprocal relationship was observed between the production of PA and of PG. This suggests that water and glycerol compete for the same phosphatidyl unit. The high concentrations of glycerol required by the PG forming enzyme (Fig. 7,10,15) are similar to those previously reported for plant PLD catalysed alcohol transfer (226,34,200). Yang et al. (226) reported that 1.1 M glycerol produces an equal rate of PG and PA formation by cabbage PLD while Dawson (34) observed

that 0.5 M glycerol was required for an equal rate of transfer and hydrolysis to occur. These values are quite similar to those observed for rat brain PG formation which was about 0.35 M (Fig. 7). This high K_m observed for the production of PG by the enzyme in brain extracts is a characteristic which argues against it having physiological significance as a route for PG formation. The concentration of glycerol is known to be in the μM range in mammalian tissues (122). The other exchange reactions of rat brain in contrast are found to have very high affinities for their substrates. The reported K_m's are for choline exchange, 190μM (197); for ethanolamine exchange, 43μM (197); for serine exchange, 15μM (197); and for inositol exchange, 11μM (14).

Phosphatidylglycerol contains 2 asymetric carbons, marked with asterisks in the diagram, and therefore theoretically can exist in four stereoisomeric forms.



3-sn-phosphatidyl-1[1]-sn-glycerol

Because the biosynthetic enzymes producing this lipid are stereospecific, a single stereoisomer should occur (111) (33). This was confirmed by Haverkate and Van Deenen (77) for PG purified from spinach leaves. The technique they used for this analysis consisted of generating glycerol phosphate

from the PG with phospholipase C, or by phospholipase D followed by alkaline hydrolysis of the resulting phosphatidic acid. The configuration of these glycerol phosphates were then determined, based upon reactivity with the stereo-specific enzyme sn-glycerol-3-phosphate dehydrogenase (77). They found that, as predicted by the pathway of Kiyasu et al. (111), spinach PG had the 1,2-diacyl-sn-glycerol-3-phosphoryl-1^1-sn-glycerol configuration. Joutti and Renkonen (98) applied this approach to the analysis of the PG produced by plant PLD catalysed glycerol exchange reaction. They found that the glycerol phosphate released by phospholipase C treatment was 50% of the sn-glycerol-3-phosphate configuration. This indicated that the original PG consisted of a racemic mixture of 1,2-diacyl-sn-glycerol-3-phosphoryl-1^1 and 3^1-sn-glycerol. The configuration of the phosphatidyl unit of PG can be assumed to have remained unaltered during PLD's action, since it is not involved in the reaction.

The stereochemistry of the PG produced by the rat brain enzyme was determined using these techniques. Approximately 35% of the glycerol phosphate liberated by phospholipase C treatment was found to have the sn-glycerol-3-phosphate configuration, indicating that the PG forming activity observed in the detergent extracts produces a racemic mixture of PG. This racemic product is a further indication that the de novo pathway is not involved in the observed PG synthesis. Such a non-stereospecific reaction is however consistent with the rat brain PLD being responsible for the PG formation.

5.2 Characteristics of PLD and its Distribution in Rat Tissue

PLD from mammalian tissues has been characterized in a detergent solubilized form in an initial extract (169) and also after partial purification (195). No examination however has been made of this enzyme while still membrane bound. The aim of this part of the study was to carry out investigations of the enzymatic characteristics of PLD prior to its detergent solubilization. Some specific questions which this work attempted to answer were the following. Are the properties of PLD in subcellular fractions similar to those previously described for the detergent solubilized enzyme? Which tissues and subcellular fractions contain the enzyme and do its properties vary with its location?

A rather dramatic difference in properties was immediately noted between the enzyme in detergent extracts and the enzyme in a non-solubilized state. As seen in Fig. 11c the enzyme is undetectable when the substrate, a $[^3H]$ phosphatidylcholine liposomal suspension and microsomes are co-incubated. PLD activity however does become detectable after the addition of a bile salt, taurodeoxycholate. This complete inactivity and stimulation by bile salt was also observed with the lung microsomal fraction. As with most enzymes affected by detergents biphasic curves were observed (62), in which the detergent caused inhibition at above optimal concentrations. The effect of varying the substrate, $[^3H]$ PC, on the brain and lung activities was examined and found to be similar in both cases. The optimal

concentration was observed to be 5 mM, above which substrate inhibition occurred. The optimal pH, in both lung and brain tissue was found to be 6.5. This value is very similar to that reported for the solubilized enzyme, which was pH 6.0 (169). The microsomal PLD was similar to the solubilized form (169) in not being inhibited by the presence of EDTA, thus indicating a lack of divalent cation requirement.

A tissue survey was carried out to determine the relative activity of PLD in different rat tissues. Such information may be useful in determing the physiological purpose of this enzyme. If it is required for the general metabolism necessary for cellular function, then it would be expected to occur in all tissues. On the other hand, if PLD has very specialized functions related to certain tissues, it may have a restricted rather than general distribution. Two procedures were used for the assay of PLD in this study. The first was based upon the hydrolytic activity, used TDOC as an activator for the enzyme and quantitated the hydrolysis of an exogenous $[^3H]$ phosphatidylcholine substrate. The second assay procedure was based upon the PG forming activity, used oleic acid as an activator for the enzyme, and the endogenous microsomal phospholipids as substrate. All tissues displayed activity, though in varying amounts. Liver, intestinal mucosa and testis were found to be the lowest in activity, while lung, brain, adipose and heart tissues were the most active.

Using brain and lung tissue, a subcellular distribution study of PLD activity was carried out to establish the

intracellular location of the enzyme. In contrast to plant tissues where much of PLD is soluble, PLD of rat brain and lung was only present in particulate fractions. Enrichments of 2.1 and 3.9 fold over the homogenates were observed with brain and lung microsomal (P_3) fractions respectively, suggesting primarily a microsomal location for this enzyme. The microsomal marker enzyme NADPH-cytochrome C reductase and PLD were observed to distribute similarly, further suggesting PLD is a microsomal activity.

Much evidence is available to indicate that essentially all microsomal vesicles have the same orientation relative to the original intact endoplasmic reticulum. That is with the outer surface corresponding to the original cytoplasmic surface and the inner surface corresponding to the original lumenal surface of the endoplasmic reticulum. One technique to analyse which surface of these vesicles an enzyme is located on makesuse of the impermeability of these membranes to macromolecules and to low molecular weight charged substances (139). By treating microsomes with proteases or nonpermeant inhibitors it is possible to infer the sidedness of enzymes in these membranes by the sensitivity of their activities to these treatments. This procedure can be used in conjunction with a recently developed technique which uses low concentrations of a detergent, deoxycholate, DOC, to make microsomes reversibly permeable, without causing structural disruption (114). An enzyme activity which is not sensitive to a protease in intact microsomes and therefore supposedly lumenally located, should be inactivated by the protease in

DOC disrupted microsomes.

The results of studies to determine the sidedness of PLD in rat brain microsomes using trypsin, pronase and PCMPS are in fairly close agreement. In intact microsomes trypsin inactivated about 24% of the PLD activity, pronase about 39% and PCMPS about 18%. After microsomes were made permeable using the DOC procedure (138), trypsin inactivated PLD by 52%, pronase by 69% and PCMPS by 91%. These results suggest that about 50% of PLD is exposed at the outer microsomal membrane since this was the maximal percentage inactivated. DOC disruption of the microsomal vesicles, allowed further inactivation of PLD. This may indicate that there are two populations of PLD, one on each side of the microsomal membrane or alternately, the DOC may simply be exposing PLD which previously had been buried within the membrane bilayer. It may also be that PLD is a transmembrane protein. Most microsomal enzymes however have been found to be asymmetrically distributed on the membrane.

5.3 Characteristics of Phospholipase D Towards in situ Microsomal Membrane Phospholipids

The aim of these experiments was to examine the reason for the detergent dependency of the microsomal PLD. This property was observed with both the brain and lung enzymes when assayed with an exogenous substrate, consisting of a $[^3H]$ phosphatidylcholine liposomal preparation. There are several possible explanations for this observation. Firstly, the enzyme may be lumenally located within the microsomal

vesicle and therefore unable to interact with the exogenous [^{3}H] PC substrate, unless a detergent disrupts the microsomal membrane. This explanation however does not seem to apply here, since the experiments with proteases and PCMPS indicated that about 50% of PLD is exposed to the outer surface. With the assumption that the active site of this enzyme is included in this exposed portion of the enzyme, about 50% of this enzyme's activity should be observable without detergents.

A second possible explanation for the lack of PLD activity might be that the liposomal [^{3}H] PC preparation used as the substrate is not recognized by the enzyme. The modification of the physical appearance of lipids by detergents is an observed mechanism for detergent activation of lipid metabolizing enzymes. Another example of this are the sphingolipid hydrolases, which are also activated by bile salts (19).

If the latter explanation is correct, that the bile salt acts to modify the physical properties of the artificially prepared membranous substrate, then the latency of this enzyme is actually an artifact of the assay system being used, rather then a property of the enzyme. On the other hand, if PLD could be demonstrated to also be inactive towards in situ microsomal membrane phospholipids, then this would suggest that an actual characteristic of the enzyme or its natural substrate is being demonstrated.

In order to measure the activity of PLD towards the in situ phospholipids of the microsomes containing the enzyme, use was made of the PG forming activity of this enzyme. It

can be seen, Fig. 10 that PLD catalysed PG formation in the presence of saturating amounts of glycerol, occurs at a similar rate as does PLD catalysed hydrolysis without glycerol present, indicating the validity of this approach. The results of this study showed a basal activity by PLD of about 0.5 $nmol \cdot mg^{-1} \cdot hr^{-1}$ in the absence of bile salt activators (Fig. 12). These detergents were observed to stimulate the activity of PLD towards the membrane phospholipids by about 10 fold.

The reason for the lack of PLD activity towards the membrane lipids is not apparent. It is interesting to contrast PLD catalysed polar head group exchange, which requires detergent for activity, to the base exchange activities for choline, ethanolamine and serine, all of which are fully active towards membrane phospholipids in the absence of detergents.

It has been suggested that certain membrane enzymes may be regulated by alterations of the amounts in cell membranes of certain lipids possessing detergent properties (177,178,215). Examples of such enzymes are guanylate and adenylate cyclase possibly regulated by lysolecithin (178) and by free fatty acids (178) (2) and liver sialyltransferase activated by lysolecithin (179). Considering that PLD may also be a membrane enzyme regulated in this way, and that bile salts do not occur in brain a range of amphiphilic lipids endogenous to that tissue were tested for their ability to activate PLD. From the selection of amphiphiles, tested at various concentrations, it was observed that only oleic acid

resulted in significant activation while slight stimulation was seen with phosphatidic acid.

Various synthetic detergents representing anionic, cationic and neutral detergent types all failed to activate the enzyme, indicating that PLD possess rather specific structural requirements for an activator.

The characteristics of fatty acid activation were studied in more detail in order to help understand their mechanism of action. The mono-unsaturated species of chain length C_{16}, and C_{18} were the best activators, being about 10 fold more potent than the bile salts, resulting in at least a 100 fold activation of PLD. The polyunsaturated C_{18} species, linoleate and linolenate, and the C_{20} polyunsaturated species arachidonate were also good activators. The C_{18} saturated fatty acids, in contrast to their unsaturated analogues, were completely ineffective, as were oleyl alcohol and methyl oleate. Laurate, a C_{12} saturated fatty acid was able to activate the enzyme. This pattern of effectiveness correlates with the detergent properties of these compounds (207). The concentration of oleic acid required for maximal activation was found to depend directly on the amount of microsomal membrane protein present in the incubations. It is not possible to decide from this observation whether the activation results from interaction of fatty acid with the membrane lipids or with the enzyme, itself, in fact both interactions may be occurring (79). It does suggest that small amounts of free fatty acid, would be adequate to activate PLD, if produced within a localized area of the

membrane, possibly via the action of a phospholipase A or lipase activity. The optimal concentration of oleate was found to also depend on the amount of exogenous $[^3H]$PC in the incubation when the hydrolytic activity of this enzyme was measured. The activation of PLD does not appear to require its solubilization from the microsomes. This was indicated by the recovery of the enzyme activity in the microsomal pellet when in the presence of the optimal concentration of oleate for its activation. The requirement for an activator was found for PLD in all tissues examined, Table 8, indicating that this is a general property of this enzyme. Oleate could activate the enzyme in all tissues, indicating that this lipid has the potential to modulate PLD in most rat tissues.

The properties of PLD catalysed PG formation were found similar in microsomes stimulated with oleate to those for the enzyme in a solubilized partially purified state. The apparent K_m for glycerol was found to be 130 mM, similar in magnitude to that found for the solubilized enzyme, which was 200 mM. This observation again suggests that the PG forming activity of PLD would not be significant at physiological concentrations of glycerol (122).

The mechanism by which fatty acids activate the microsomal PLD is not apparent. A number of possible explanations can be considered. For example it is possible that these lipids may act by bringing about increases in membrane fluidity (175,159). This might enhance diffusion of substrates to and products away from the enzyme, or possibly affect the activity

of the enzyme itself. Such a mechanism for the activation of calf brain neuraminidase by the anesthetics nitrous oxide and halothane has been suggested (173). These anesthetics which were shown to increase membrane fluidity activated that microsomal enzyme towards membrane bound substrates but not towards a soluble substrate sialyllactitol. Thus it was considered that this fluidity change could result in increased lateral diffusion of the membrane bound substrate and so increase the rate of its reaction (172). It is also quite possible that the action of the fatty acids involves their direct interaction with the enzyme. This possibility is suggested by the reports of a number of soluble enzymes acting on water soluble substrates, which are activated by fatty acids. These are guanylate cyclase of platelets (65), glycogen phosphorylase kinase of rabbit skeletal muscle (181), calmodulin dependent phosphodiesterase of brain (219), choline phosphate-CTP cytidylyltransferase of lung (214) and bee venon phospholipase A_2 (46).

Continuing work will be necessary to establish the mechanism of activation of PLD by free fatty acids. It seems that further investigations of this problem may lead to an understanding of how PLD is regulated in mammalian systems. Such a study of PLD would no doubt contribute to the more general problem of the properties and regulation of membrane bound enzymes acting on lipid and other substrates that are components of the same membrane.

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