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(Signed)....Jung-shou Chen.....

PERMANENT ADDRESS:

.....Dept. of Biochemistry
....University of Ottawa
....Ottawa, Ontario

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THE UNIVERSITY OF ALBERTA

STUDIES ON PHOSPHOLIPASE D

by

© JUNG-SHOU CHEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1972

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "STUDIES ON PHOSPHOLIPASE D" submitted by JUNG-SHOU CHEN in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Peter G. Pearson
Supervisor

Ronald N. McElhaney

Neil Madsen

John Ho

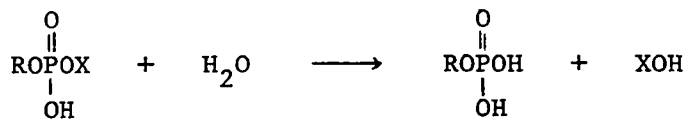
A. C. Morgan

External Examiner

Date January 11, 1972

ABSTRACT

The enzyme phospholipase D (E.C. 3.1.4.4) which occurs in several plants, is known to catalyze the following reaction:



where R is 1,2-diacyl-sn-glycerol or a monoacyl-sn-glycerol and X is choline, ethanolamine, serine or glycerol. In the case of lecithin substrates this reaction can be conveniently followed by measuring the liberation of choline.

Previous work had indicated that the enzymic reaction was highly dependent on the physical structure of the substrate and particular emphasis had been placed on the electrostatic properties of the lipids. In the work reported in this thesis, an attempt was made to evaluate more precisely the influence of other physical properties on the enzymic reaction.

In order to avoid side-reactions which might lead to choline formation by a different enzymic route, substrate and product analogs possessing non-hydrolysable hydrocarbon substituents were sought. The compounds synthesized were 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine, 1,2-dihexadecyl-sn-glycero-3-phosphorylcholine, 1,2-ditetradecyl-sn-glycero-3-phosphorylcholine and the corresponding 1,2-dialkyl-sn-glycero-3-phosphoric acids. The 1,2-dialkyl-sn-glycero-3-phosphorylcholines were obtained by phosphorylation of the corresponding 1,2-dialkyl-sn-glycerols with monophenylphosphorodichloridate and subsequent reaction with

choline iodide. 1,2-dialkyl-sn-glycero-3-phosphoric acids were prepared by phosphorylation of the corresponding 1,2-dialkyl-sn-glycerols with diphenylphosphorochloridate and subsequent catalytic hydrogenolysis. 1,2-dimethyl-sn-glycero-3-phosphorylcholine and 1,2-dioctadecyl-sn-glycero-3-(methyl)phosphorylcholine were also prepared.

1,2-dialkyl-sn-glycero-3-phosphorylcholines were found to be poor substrates for phospholipase D when compared with egg-lecithin. Diethyl ether, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTABr), a nonionic detergent (BRIJ 58) and several phosphatidic acids were then used to modify the physical structure of the substrates.

First, a turbidimetric method was used to examine the effects of SDS, CTABr and BRIJ 58 on the structure of the lipid-water system. All the dialkyl ether phospholipid dispersions were progressively cleared by increasing amounts of each of these compounds. The higher homologues required more of each detergent to produce a given decrement in absorbance than did the lower homologues. At the same time SDS, BRIJ 58 and certain phosphatidic acids activated the enzymic hydrolysis whereas CTABr was a potent inhibitor. Secondly, the technique of differential thermal analysis (DTA) was used to examine the thermotropic and lyotropic mesomorphism of the phospholipids and phospholipid:SDS mixtures. SDS decreased the transition temperatures of the dialkyl ether phospholipids in excess water in a manner which correlated well with the susceptibility to enzymic hydrolysis. Comparison of the optimal conditions of concentration and temperature

for enzymic hydrolysis of the ether lipids with those required for hydrolysis of egg lecithin and hydrogenated egg lecithin led to the conclusion that the substrate must be in a liquid crystalline state in order for hydrolysis to commence.

The results have been interpreted in terms of four factors which could control the rate of enzymic hydrolysis:

1) the interfacial area which is available for adsorption of the enzymic protein
2) the ability of the enzyme to penetrate between the polar head groups of the lipids prior to attack on the phosphodiester bond
3) the fluidity of the hydrocarbon chains of the lipid molecules which may be necessary for hydrophobic interaction between the lipid and protein components
4) the sign and magnitude of the electrostatic charge on the lipid surface which may control the initial approach of the enzyme to the substrate.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and thanks to my advisor, Dr. P.G. Barton, whose endless patience and constructive comments were an invaluable aid during the progress of this research. The many hours of discussion and helpful advice provided by Drs. R.N. McElhaney, W.C. Day and other members of this Department are also appreciated.

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LIST OF ABBREVIATIONS

14C EPC	1,2-ditetradecyl-sn-glycero-3-phosphorylcholine
14C EPA	1,2-ditetradecyl-sn-glycero-3-phosphoric acid
14C Na_2EPA	disodium salt of 14C EPA
16C EPC	1,2-dihexadecyl-sn-glycero-3-phosphorylcholine
16C EPA	1,2-dihexadecyl-sn-glycero-3-phosphoric acid
18C EPC	1,2-dioctadecyl-sn-glycero-3-phosphorylcholine
18C EPA	1,2-dioctadecyl-sn-glycero-3-phosphoric acid
SDS	sodium dodecyl sulfate
CTABr	cetyltrimethylammonium bromide
BRIJ 58	nonionic detergent, polyoxyethylene cetyl ether
egg PC	egg yolk phosphatidylcholine, egg yolk lecithin
egg PA	phosphatidic acid derived from egg PC
egg Na_2PA	disodium salt of egg PA.

CHAPTER I

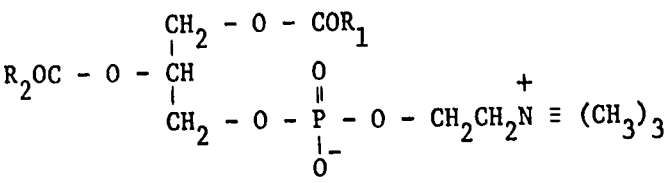
GENERAL INTRODUCTION

The studies reported in this thesis deal mainly with the chemical synthesis and enzymic hydrolysis of dialkyl ether phospholipids. The background to these studies is discussed in four categories. The first comprises an introduction to the chemical synthesis of phosphatidylcholines, phosphatidic acids and their ether analogues. Secondly, the distribution and biosynthesis of the ether phospholipids are reviewed briefly. Thirdly, the physical structure and behavior of phospholipids are discussed briefly. Fourthly, some properties of the phospholipases are described.

A. Chemical Synthesis of Phospholipids

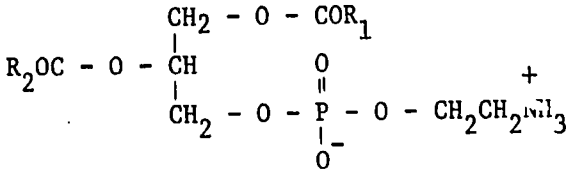
The chemical synthesis of phospholipids has played an important part in establishing the chemical structures of lipids. The pioneering studies of Baer and his associates (see 1) contributed significantly to the assignment of the L-α configuration to lecithins and other phospholipids from higher animals. Secondly, the isolation of naturally occurring phospholipid leads to preparation of sub-classes containing numerous homologues differing in their fatty acid constituents. Therefore, the synthetic phospholipids may serve as references and model substances in analytical, biochemical and biophysical studies. The chemical structure of certain phospholipids is shown in Fig. 1.

Figure 1. Chemical structure of some phospholipids.

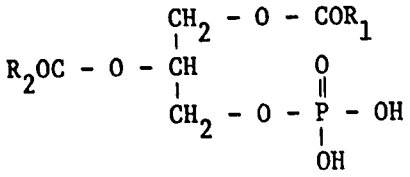


Phosphatidyl choline

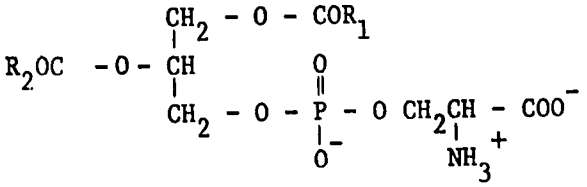
(Lecithin)



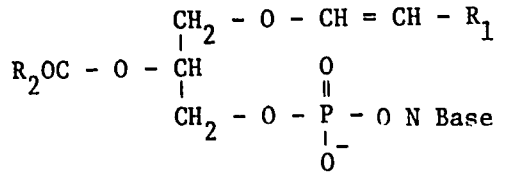
Phosphatidyl ethanolamine



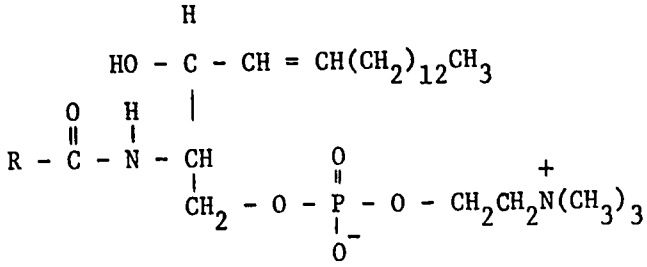
Phosphatidic acid



Phosphatidyl serine



Plasmalogen (N base = choline
or ethanolamine)



Sphingomyelin

1. Chemical Synthesis of Diacyl Phospholipids

Within reasonable limits of space, it is not possible to survey the chemical syntheses of all phospholipid types. Since the phospholipids used in this work are phosphatidylcholines, phosphatidic acids and their dialkyl ether analogues, this introduction will be concerned only with syntheses of phosphatidylcholines and phosphatidic acids.

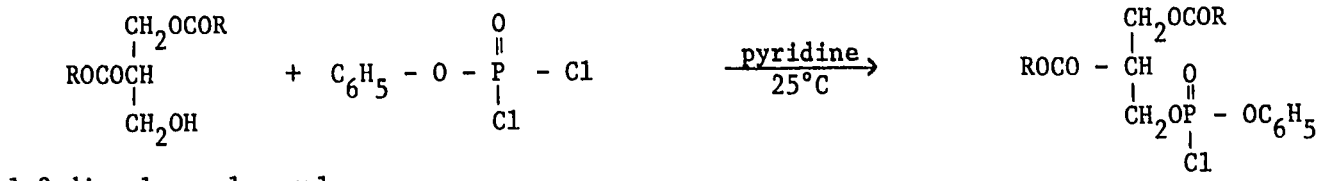
(a) Fully Saturated Lecithins

The synthesis of enantiomeric forms of lecithins was first described by Baer and Kates (1) in 1950. They reported the phosphorylation of an α,β-diglyceride with monophenylphosphoryl-dichloridate in the presence of pyridine and subsequent reaction with choline chloride to yield a diacyl-L-α-glycerylphosphorylcholine chloride which was then separated via a Reinecke salt. Subsequent removal of chloride ion and hydrogenolysis yielded the fully saturated L-α lecithin. An improved simplified procedure for the synthesis of saturated lecithins was reported by Baer and Marukas (2). This procedure was also applied to the synthesis of water-soluble lecithins containing short chain fatty acids (3). This is described in part by scheme 1. Malkin and Bevan (4) found it advantageous to use choline iodide instead of the extremely hygroscopic chloride.

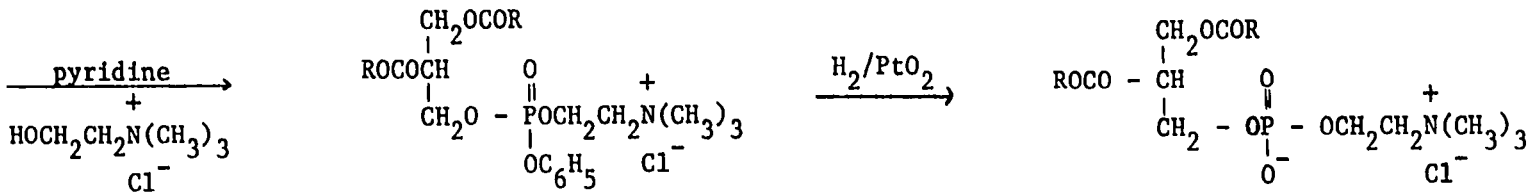
(b) Unsaturated Lecithins

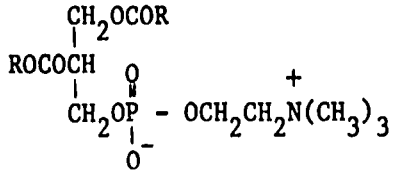
It is obvious from the above synthetic approach that the use of hydrogenolysis for removal of the phenyl group does not permit the synthesis of unsaturated lecithins. Baer, Buchnea and Newcombe (5) achieved the first synthesis of a fully unsaturated L-α-lecithin,

Scheme 1. Synthesis of fully saturated lecithin (Reference 2).



1,2-diacyl-sn-glycerol





1,2-diacyl-sn-glycero-3-phosphorylcholine

(L-α-lecithin)

L-α-(dioleyl)-lecithin. The reaction is shown in scheme 2. Baer (6) achieved an alternative route for the preparation of dioleyl-L-α-lecithin by the phosphorylation of D-α,β-diolein with phosphorus oxychloride in the presence of quinoline. The resulting phosphatidic acid dichloride was esterified with choline iodide and the reaction mixture separated by column chromatography on silicic acid.

(c) Indirect Synthesis

Tattrie and McArthur (7) achieved the acylation of L-α-glycerylphosphorylcholine (L-α-GPC) with acyl chloride in anhydrous chloroform. A similar preparation of various lecithins was reported by Kögl et al. (8). Baer and Buchnea (9) significantly improved the preparation of L-α-lecithin by acylation of the cadmium chloride adduct of GPC in the presence of pyridine and both saturated and unsaturated L-α-lecithins were obtained in a good yield.

(d) Mixed Acid Lecithin

The synthesis of lecithin containing two different fatty acids in different positions has been accomplished by two different methods.

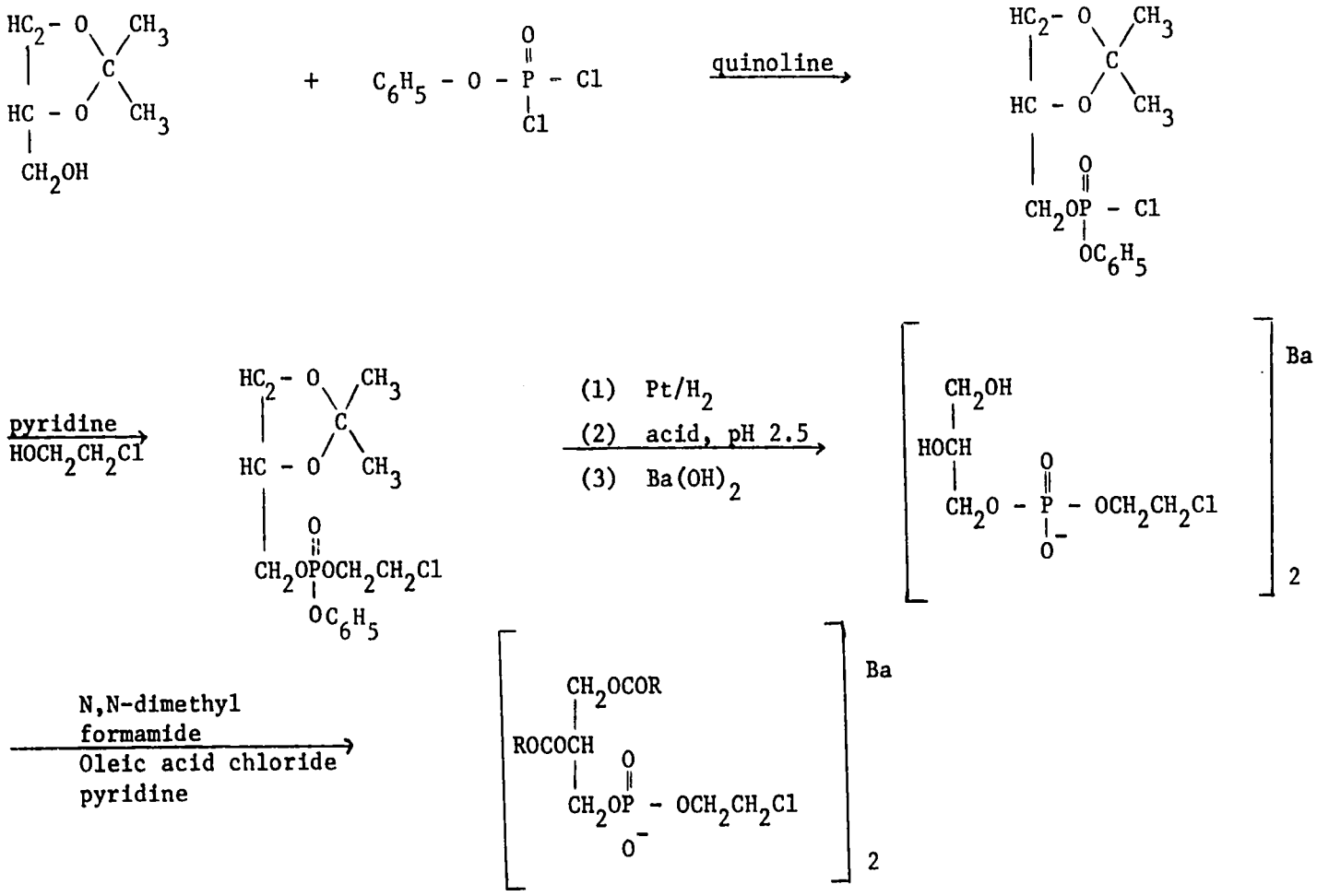
(i) Synthesis de novo of mixed-acid lecithins

Various enantiomeric β,γ-diacylglycerol-α-iodohydrins have been prepared by de Haas and van Deenen (10) and these compounds converted into the lecithins as shown in scheme 3.

(ii) Partial synthesis of mixed-acid lecithins

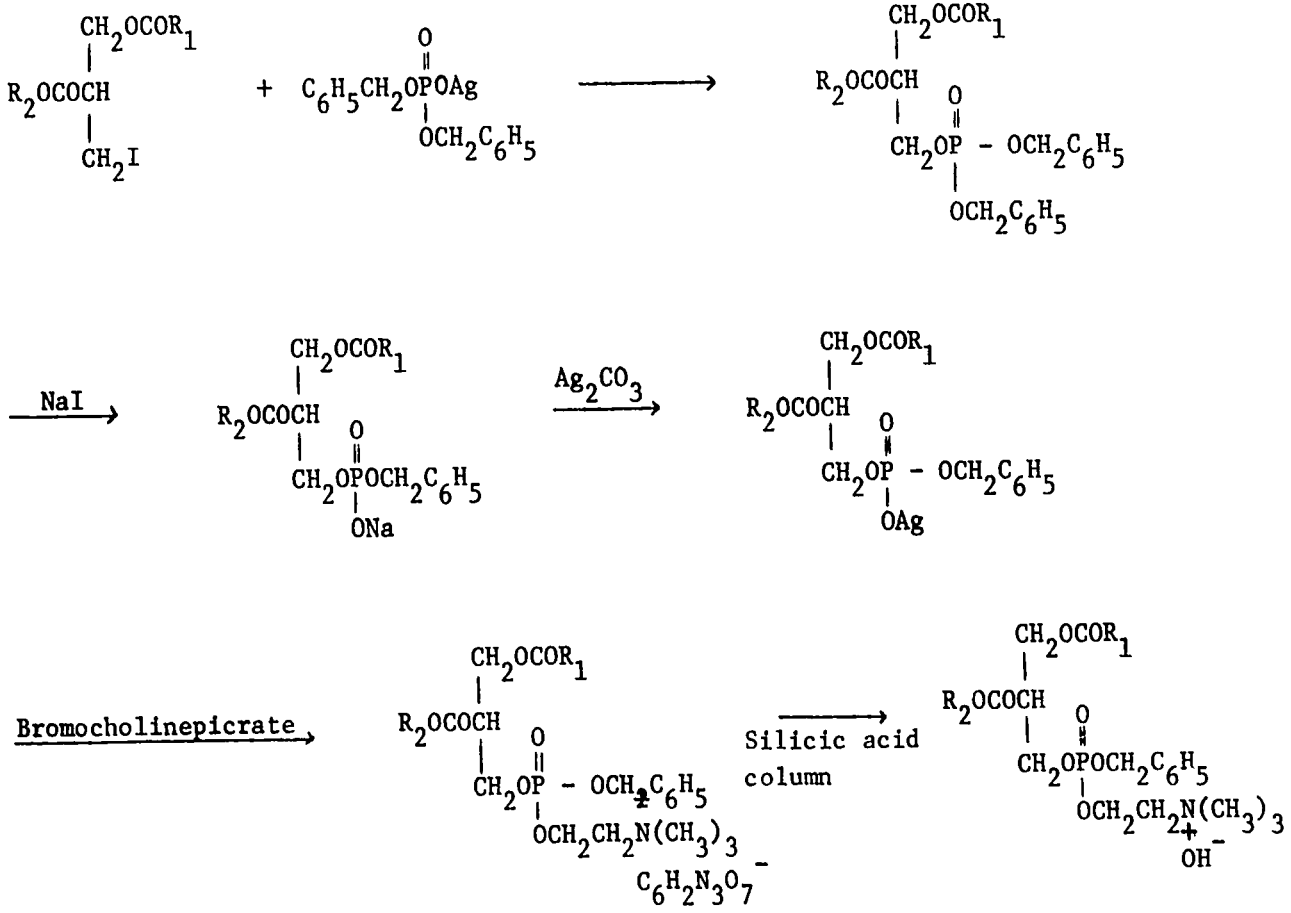
This approach was utilized by de Haas and van Deenen (11) and by Hanahan and Brockerhoff (12). In principle, an L-α-lecithin containing two identical fatty acids, readily prepared from L-α-GPC, is subjected to a hydrolysis catalyzed by phospholipase A_2. The

Scheme 2. Synthesis of unsaturated lecithin (Reference 5).



1,2-dioleyl-sn-glycero-3-phosphorylcholine

Scheme 3. Synthesis of mixed-acid lecithin (Reference 11).


NaI
Ag2CO3
Bromocholinepicrate
Silicic acid column
H2/Pd
1-acyl(R1)-2-acyl(R2)-sn-glycero-3-phosphorylcholine

resulting γ-acyl derivative is then reacylated with the halide of a second fatty acid.

(e) Phosphatidic acids

Baer (13) prepared a number of structurally and optically pure L-α-phosphatidic acids by phosphorylation of the D-α,β-diglycerides with diphenylphosphorylchloride and removed the phenyl groups from the phosphorylated product with Adams' catalyst. However, the compounds possessed irreproducible melting points. This was attributed to the presence of small amounts of alkali in the Adams' catalyst causing partial salt formation of the phosphatidic acids. Uhlenbrock and Verkade (14) also prepared racemic α-phosphatidic acids and β-phosphatidic acids by a similar method. Baer and Buchnea (15) prepared an unsaturated phosphatidic acid by phosphorylation of D-α,β-diolein with phosphorus oxychloride. A convenient method for the preparation of saturated phosphatidic acids was demonstrated by Verkade's group (16). They phosphorylated an α-iododiglyceride with silver dibenzyl phosphate and removed the benzyl group by hydrogenolysis. Baylis et al. (17) and Stanacev and Kates (18) also used the same method to prepare optically pure L-α-phosphatidic acids. An unsaturated L-α-phosphatidic acid of mixed-acid type was prepared by de Haas et al. (19) by a reaction between a mixed-acid diacyl glycerol-L-α-iodohydrin and silver dibenzyl phosphate followed by protection of the double bond with bromine at -20°C. After removal of the benzyl group by hydrogenolysis, bromine was removed with activated zinc dust. Recently, Bonsen and de Haas (20) reported on the synthesis of silver-di-t-butyl phosphate and its reaction with diacyl glycerol-3-iodohydrin to

give the protected phosphatidic acid. The protecting group was removed under acidic conditions which do not affect the double bond of the unsaturated fatty acids. Lipidot et al. (21) have described the synthesis of α-DL (and L)-diacylglycerol phosphate which involved a direct acylation of glycerophosphate by fatty acid anhydride in the presence of the appropriate tetramethylammonium salt. The procedure is a general one and can be used for the synthesis of saturated and unsaturated phosphatidic acids.

2. Chemical Synthesis of Ether Analogues of Phospholipids

(a) Dialkyl Ether Analogues of Lecithin and Phosphatidylethanolamine

1,2-dialkyl glycerol ethers with two identical alkyl moieties and with two different alkyl moieties have been synthesized by specific alkylation of glycerol or 1-O-alkyl glycerol, in which the OH group in position 3 had been protected by a benzyl or trityl group (22-28). An improved synthesis of 1,2-dialkyl glycerol ethers was described by Paltauf and Spener (29) who started from 3-tetrahydropyranyl-glycerol and alkyl methanesulfonates. Both saturated and unsaturated dialkyl ether glycerols could conveniently be prepared. The first synthesis of a dialkyl ether analogue of lecithin was reported by Stanacev et al. (30). They obtained 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine by phosphorylation of 1,2-dioctadecyl-sn-glycerol with phenylphosphoryldichloridate, and subsequent reaction with choline iodide and then Ag_2CO_3 followed by catalytic hydrogenolysis to remove the protecting phenyl group. Rosenthal and his coworkers (31-34) synthesized a variety of racemic ether analogues of phospholipids and Chacko and Hanahan (28)

prepared optically active diether analogues of lecithin and phosphatidylethanolamine by phosphorylation of di-O-alkyl glycerols such as 1,2-di-O-cis-9'-octadecenyl-sn-glycerol with $POCl_3$. Chen and Barton (35) have described the synthesis of 1,2-dialkyl-sn-glycero-3-phosphoric acids by phosphorylation of the corresponding 1,2-dialkyl-sn-glycerols with diphenylphosphorochloridate and subsequent catalytic hydrogenolysis to remove protecting phenyl groups. Kates and his associates (36) have carried out the synthesis of phytanyl diether glycerol and diasterioisomers of di-O-phytanyl ether analogues of phosphatidylglycerol phosphate and phosphatidylglycerol. In both cases, the 2,3-di-O-phytanyl-1-glycerophosphoryl-3'-glycero-1'-phosphate and the 2,3-di-O-phytanyl-1-glycerophosphoryl-3'-glycerol isomers were identical with the respective natural isomers isolated from H. cutirubrum (36). Recently, they also reported the synthesis of diphytanyl ether analogues of phosphatidic acid and cytidine diphosphate diglyceride (37).

(b) Monoalkyl Monoacyl Analogues of Phosphatidylcholine and Phosphatidylethanolamine

Chemical synthesis of racemic phospholipids of this class was first achieved by Malkin's group (38-39). Chacko and Hanahan (28) have prepared this type of compound starting with a synthetic monoalkyl monoacyl glycerol. Recently, they also reported the synthesis of phosphonic acid monoether analogues of phosphatidylcholine and phosphatidylethanolamine (40).

(c) Alk-1-enyl-ether Phospholipids

Slotboom, de Haas and van Deenen (41) have described the partial synthesis of a natural plasmalogen. They used the cis-1-O-(alk-1'-enyl)-sn-glycerol derived from ox heart plasmalogens by hydrolysis with phospholipase C followed by alkaline hydrolysis to remove β-fatty acids. This alk-1-enyl glycerol was converted by acylation to the 2,3-dioleoyl derivative which on treatment with pancreatic lipase gave 2-oleoyl-cis-1-O-(alk-1'-enyl)-sn-glycerol. This was then converted to a plasmalogen by reaction with 2-bromoethylphosphoryldichloride and trimethylamine yielding the plasmalogen with the natural configuration.

B. Glyceryl Ether Phospholipids

1. Distribution of Glyceryl Ether Phospholipids in Nature

The existence of long-chain ethers of glycerol in naturally occurring lipids has been known for many years (42-43). Phospholipid derivatives of 1-glyceryl ether have a rather wide distribution in animal tissue (44). Although in many tissues they comprise less than a few mole percent of the total phospholipids, they occur more abundantly in erythrocytes and bone marrow (45-46). In terrestrial slugs (47) and various molluscs (48), up to 25 per cent of the total phospholipid is comprised of ether derivatives. There are two classes of ether-containing phospholipids, monoether-monoester phospholipids and diether phospholipids. The monoether-monoester phospholipids known to occur naturally are mainly analogues of phosphatidylethanolamine (49-52) and phosphatidylcholine (53-54). Phospholipids containing two ether groups have been isolated from natural mixtures. Marinetti, Erbland and Stotz

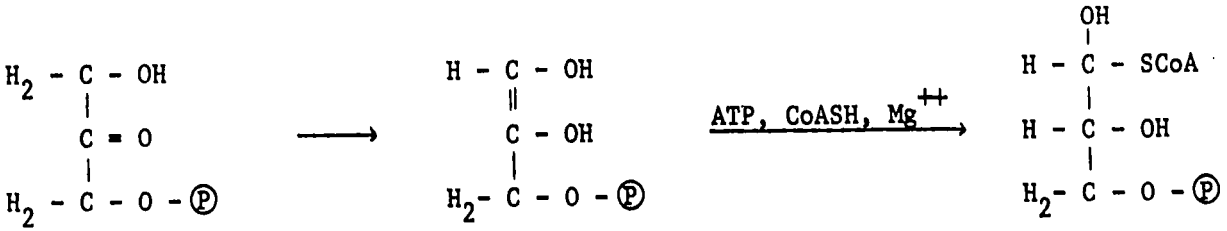
(55) obtained a phosphatide fraction from a hydrogenated mixture of beef heart phosphatides, which on acid hydrolysis yielded an α,β-dialkyl glycerol ether. Sehgal, Kates and Gibbons (56) found that the phospholipids of Halobacterium cutirubrum after acid hydrolysis also yielded a diether of glycerol. Kates, Sastry and Yengoyan (57-58) and Faure et al. (59-60) have found diether analogues of phosphatidyl-glycerol and of phosphatidylglycerol phosphate In these compounds the two alkyl units are dihydrophytyl groups. They also found that the either-containing glycerol moiety in these compounds is of the opposite stereochemical configuration to that of most natural phospholipids.

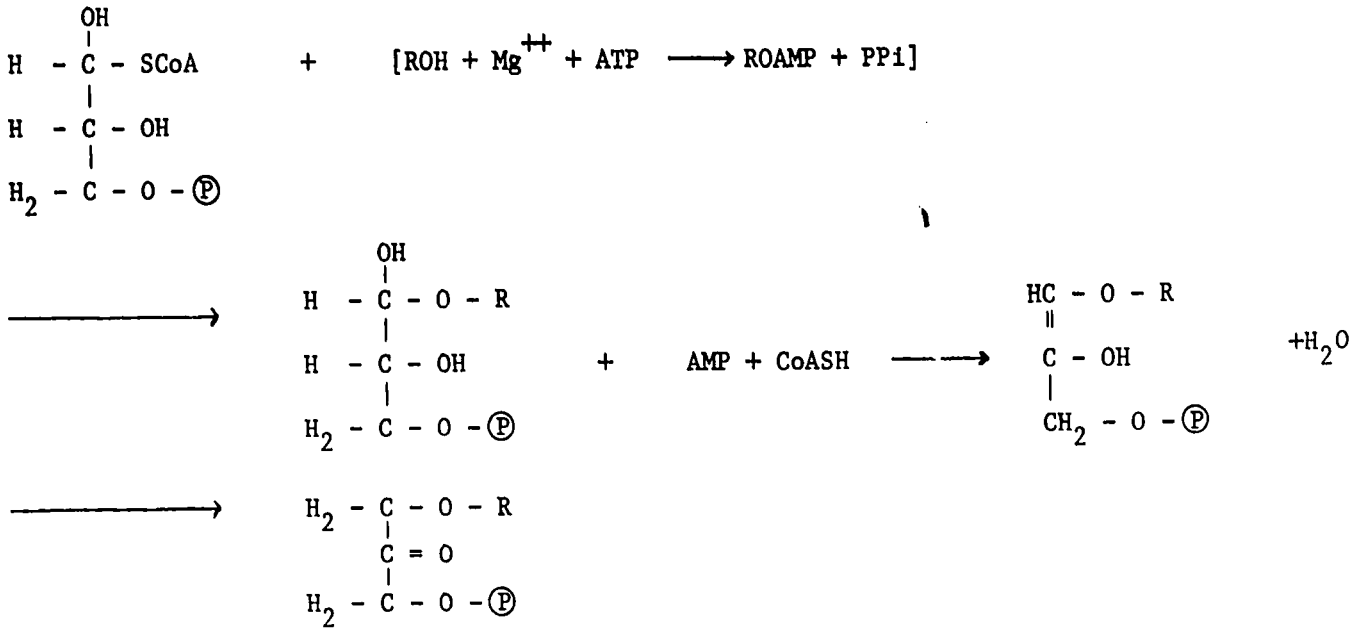
2. Biosynthesis of Ether Phospholipids

The reaction mechanism involved in the biosynthesis of the ether bond in glycerolipids is unknown but considerable progress has now been made through the use of a cell-free enzyme preparation. Snyder and his coworkers (61-69) have found that cell-free homogenates of preputial tumors as well as normal and Ehrlich ascites cells could incorporate long chain fatty alcohols into alkyl glyceryl ethers in the presence of microsomal and soluble cell fractions, as well as ATP, CoASH and Mg^{++}. They also showed that the soluble cell fraction could provide a source of the glycerol moiety (70) and this was then shown to be glyceraldehyde-3-phosphate (71). However, through the use of a specific inhibitor of triose-Ⓟ-isomerase, 1-hydroxy-3-chloro-2-propanone phosphate, Snyder's group (62) found that dihydroxyacetone-Ⓟ was the obligate triose -Ⓟ precursor. The postulated pathway for the biosynthesis of the O-alkyl linkage is shown in scheme 4 (63). Such a mechanism would permit the formation of a second ether bond in position 2 of glyceryl ethers as in

Scheme 4A. Postulated pathway for the biosynthesis of O-alkyl linkage (Mechanism I). Reference (63).

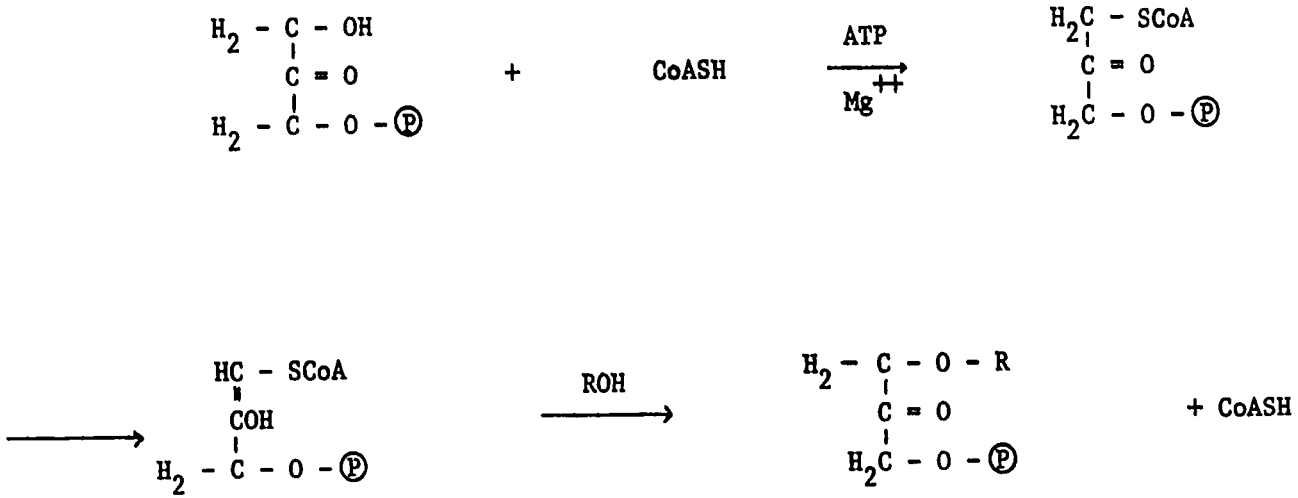
Mechanism I





Scheme 4B. Postulated pathway for the biosynthesis of O-alkyl linkage (Mechanism II). Reference (63).

Mechanism II



halophilic bacteria (56-57). A third possible mechanism for the biosynthesis of O-alkyl bond would involve attachment of DHAP to the enzyme and, while still enzyme-bound, conversion to glyceraldehyde-3-phosphate. This could then form a thiohemiacetal with CoASH before conversion to the O-alkyl compound (70). Recently, Snyder et al. (72) have reported that cytidine diphosphate choline or cytidine diphosphate ethanolamine, O-alkylacyl glycerol and Mg^{++} are essential for the biosynthesis of O-alkyl phospholipids. The postulated mechanism is shown in scheme 5 (72). Snyder et al. (66) also reported that the oxygen of ^{18}O-labeled hexadecanol was incorporated into the O-alkyl lipid in vitro and in vivo.

The mechanism of formation of alkenyl ethers is somewhat conflicting. Earlier experiments (73-75) suggested that the alkylacyl-phosphoglycerides serve as the precursor of the alkenylacylphosphoglycerides. Moreover, in vivo experiments following the metabolism of chimyl alcohol and phosphatidylethanolamine in rat brain ruled out the diacylphospho-glyceride as a precursor of 1-alkenyl-2-acylethanolamine phospho-glycerides and indicated that chimyl alcohol was incorporated into this class of lipids (76). The hypothesis proposed by Thompson (71) was that O-alkyl glyceryl ethers might be precursors of the plasmalogens. Furthermore, Keenan, Brown and Marks (78), Friedberg and Greene (79) and Wood and Healy (80) have demonstrated that the long chain fatty alcohols can be incorporated into plasmalogens more efficiently than fatty acid or fatty aldehyde. Recently, Snyder et al. (81) reported the enzymic synthesis of ethanolamine plasmalogens. Data obtained with ^{14}C-labeled dihydroxyacetone-P and

Scheme 5. Postulated mechanism for the biosynthesis of O-alkyl phospholipids (Reference 72).

$$\text{DHAP} + \text{ROH} \xrightarrow{\text{CoA, ATP, } Mg^{++}} \text{O-alkyl-DHAP} + \text{HOH} \quad (1)$$

$$\text{O-alkyl-DHAP} \longrightarrow \text{O-alkyl-DHA} + \text{Pi} \quad (2)$$

$$\text{O-alkyl-DHA} \xrightarrow{\text{NADPH}} \text{O-alkylglycerol} \quad (3)$$

$$\text{O-alkyl-DHAP} \xrightarrow[\text{acyl-CoA}]{\text{NADPH}} \text{O-alkylacyl-GP} \quad (4)$$

$$\text{O-alkylacyl-GP} \longrightarrow \text{O-alkylacylglycerol} + \text{Pi} \quad (5)$$

$$\text{O-alkylacylglycerol} \xrightarrow{\text{acyl-CoA}} \text{O-alkyldiacylglycerol} \quad (6)$$

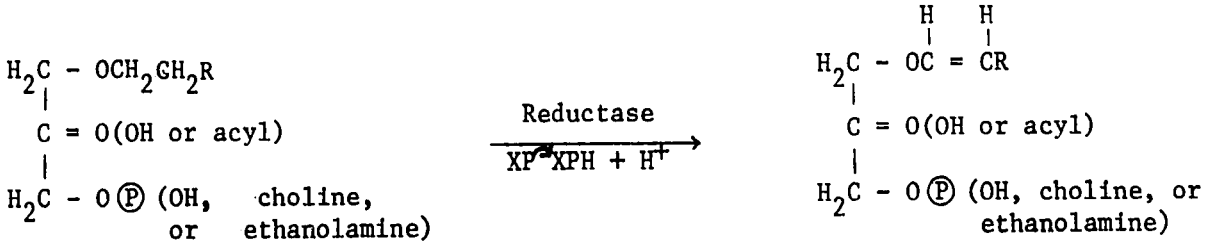
$$\text{O-alkylacylglycerol} \xrightarrow[Mg^{++}]{\text{CDP-choline}} \quad (7)$$

O-alkylacylglycerylphosphorylcholine

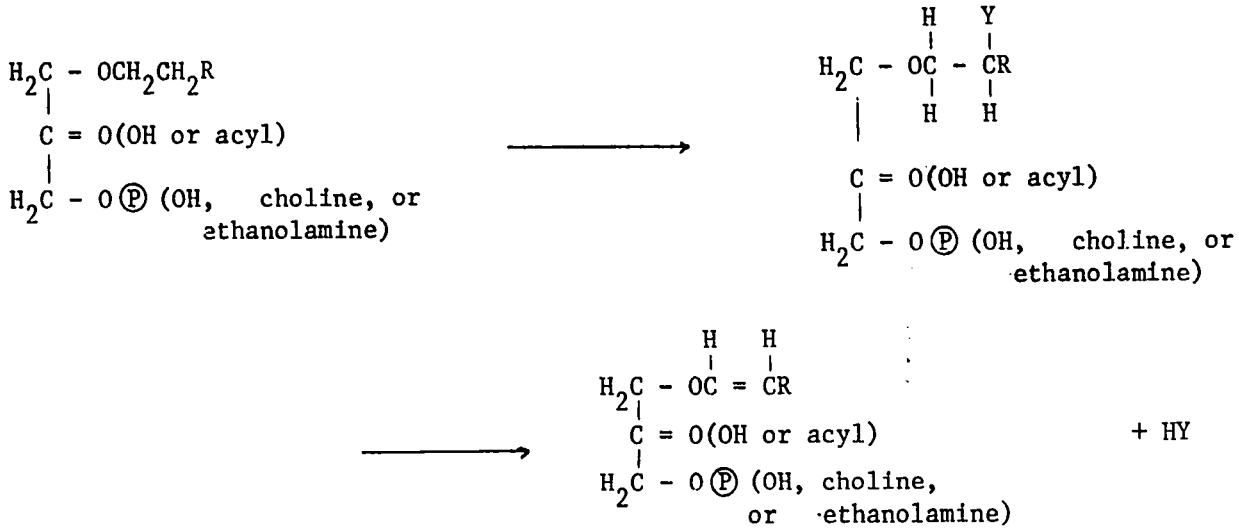
$$\text{O-alkylacylglycerol} \xrightarrow[Mg^{++}]{\text{CDP-ethanolamine}} \quad (8)$$

O-alkylacylglycerylphosphorylethanolamine

Scheme 6. Postulated reactions for the conversion of O-alkyl to O-alk-1-enyl (Reference 63).



The XP represents a hydrogen acceptor such as NAD, NADP or FAD.



The substituent "Y" on the O-alkyl moiety could be an OH, $-OCH_3$, $-OCH_2CH_3$, $-NH_2$, -SH or similar moiety.

9, 10-^{3}H hexadecanol demonstrated that the ^{3}H:^{14}C ratios in O-alkylglycerol and O-alk-1-enyl glycerol were essentially identical.

At least two possible reactions could be envisioned for the conversion of O-alkyl to O-alk-1-enyl moieties. This is shown in scheme 6 (63).

C. Physical Structure and Behavior of Phospholipids

1. Thermotropic and Lyotropic Behavior

It is well known that many organic substances do not melt simply from a crystalline solid to an isotropic liquid but instead pass through a series of mesomorphic phases before finally transforming to an isotropic liquid (82-84). These mesomorphic phases have been called "liquid crystalline" phases since they have some characteristics of both liquids and crystals. For instance, saturated 1,2-diacyl-L-α-phosphatidylcholines exhibit mesomorphism and on heating the solid lecithin, three different liquid crystalline phases can be characterized (85). The reason for this behavior is that the magnitude of the interaction forces between the hydrocarbon chains is considerably less than that of the ionic forces between the polar head groups. The hydrocarbon chains melt at a much lower temperature than do the polar head groups. The gel to liquid crystalline transition temperatures depend on the fatty acid residues of the phospholipids (85). The shorter the hydrocarbon chain length, the lower the transition temperature. If the fatty acid residues of the phospholipids are cis-unsaturated the transition occurs at much lower temperatures than with the fully saturated fatty acid residues (85).

In the lipid/water system, the liquid-crystalline transition temperature is lowered as the amount of water is increased, and finally reaches a limiting value (86-87). There are a number of important features associated with the transition temperature for the lipid when it is in the presence of water (87). The first is that the ability to disperse the lipid in water increases markedly above this temperature. Only those phospholipids which have transition temperatures below or near room temperature spontaneously form myelin figures in the presence of water. Myelin figures are tubular or occasionally globular arrangements of phospholipids organized into a bilayer structure and separated by aqueous channels. Fully saturated phospholipids which have higher transition temperatures do not form myelin figures at room temperature. In addition, they form monolayers which are more condensed than those formed by phospholipids containing cis-unsaturated hydrocarbon chains (87-89). X-ray diffraction studies also indicate a larger molecular area occupied by unsaturated lipids in three dimensional systems.

Luzzati (84) considers the following structural models to be consistent with the X-ray diffraction data:

Hexagonal type I (H_I): The structure is formed by cylinders organized into a two dimensional array (Fig. 2B). The interior of the cylinders is composed of partially disordered hydrocarbon chains while water fills the gaps between the cylinders.

Hexagonal type II (H_{II}): The lattice dimensions are similar to H_I but the water is inside the cylinders while the hydrocarbon chains form a disordered matrix between them (Fig. 2B).

The hexagonal phase shown in Fig. 2A is of this type.

Lamellar Lα, high temperature form: This phase is formed above the a-a line in Fig. 2A. The lipid molecules are associated in lamellae, filled by the hydrocarbon chains and covered by the polar head groups. The lamellae are parallel, equidistant and separated by layers of water. Thermodynamic analysis (90a) has indicated that the overall hydrocarbon chains of the phospholipid molecules are intermediate in order between crystal and bulk liquid hydrocarbon. Electron spin resonance techniques (90b, 90c) have also revealed a difference in the extent of ordering along the length of the lipid hydrocarbon chains. Those methylene groups near the carbonyl group are nearly as highly ordered as in the crystal while the order decreases exponentially toward the terminal methyl groups which resemble bulk liquid hydrocarbon.

Lamellar, Lα, low temperature form: Some of the lipid hydrocarbon chains are stiff and are hexagonally packed over part of their length.

Lamellar Lβ: This phase is obtained at very low water concentration (Fig. 2A). The hydrocarbon chains are stiff and are packed in a two-dimensional hexagonal lattice. The thin disordered layer in the middle of the hydrocarbon leaflet takes into account the length heterogeneity of the chains (Fig. 2C-2).

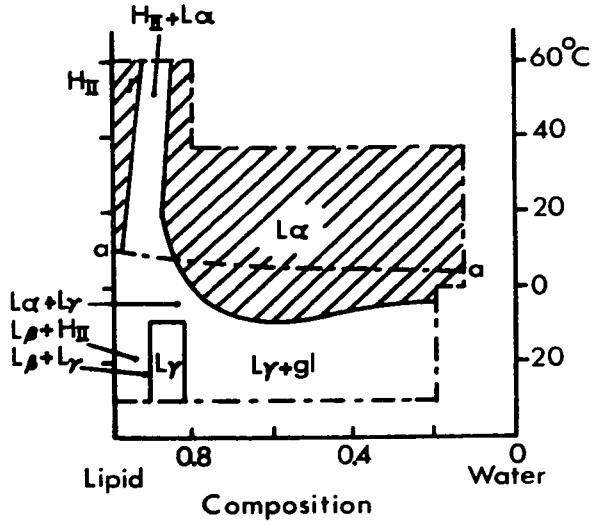
Lamellar Lγ complex: This phase is observed at low temperature over a narrow concentration range ($0.9 > C > 0.8$, Fig. 2A). It is formed by the alternate sequence of Lα and Lβ leaflets. Lα is in the low temperature form.

Figure 2A. Phase diagram of the system containing mitochondrial lipids and water. The region explored is defined by the dotted line (Reference 84).

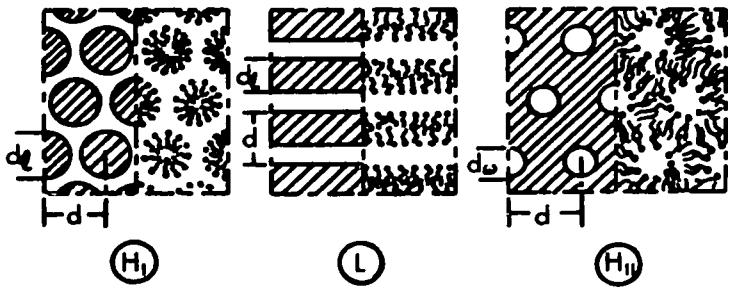
Figure 2B. Proposed structure of some "liquid-paraffin" phases. The hydrophilic groups of the lipid molecules are represented by dots, the hydrocarbon chains by wriggles. H_I: hexagonal type I. H_{II}: hexagonal type II. L: lamellar (Reference 84).

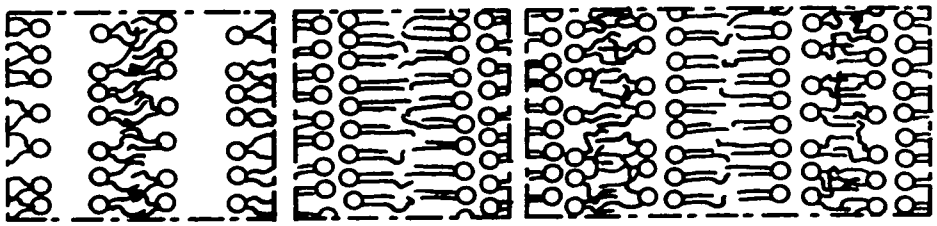
Figure 2C. Proposed structure of the various lamellar phases. The hydrophilic groups are represented by circles, the hydrocarbon chains by lines. 2C - 1: Lα; high-temperature form. 2C - 2: Lβ; 2C - 3: Lγ (Reference 84).

2A


H_{II}+Lα
H_{II}
60°C
40
20
0
20
Lα
a
a
Lα+Lγ
Lβ+H_{II}
Lβ+Lγ
Lγ
Lγ+gl
0.8
0.4
0
Lipid
Water
Composition

2B


d_ℓ
d
d_ω
d
d
H_I
L
H_{II}


2C-1
2C-2
2C-3

2. Electrostatic Environment of the Lipid-water Interface

Phospholipid molecules at interfaces orientate themselves in such a way that the polar groups are directed towards the aqueous phase and the hydrocarbon chains towards the interior of the particles. The electrostatic charges on the head groups attract oppositely charged counterions in the bulk aqueous phase. Thus, the distribution of these counterions will determine the potential gradient from the plane of the head groups into the aqueous phase. The total potential difference is known as the surface potential (ψ_G). The magnitude of this potential can be calculated from the Gouy equation (91).

$$\psi_G = \frac{2kT}{\epsilon} \sinh^{-1} \left(\frac{134}{A c_1^{1/2}}\right)$$

where ψ_G is in millivolts, $\frac{2kt}{\epsilon}$ = 50.4 mv at 20°C. A is the area in Å^2 per electronic charge and C_1 is the total concentration of the uni-univalent electrolyte in the bulk aqueous phase.

The potential at the plane of shear between the phospholipid particles with its associated counterions and the bulk aqueous phase is defined as the zeta potential (ζ). The zeta potential can be calculated from the Helmholtz-Smoluchowski equation (92).

$$\zeta = \frac{4\pi\eta}{D} \cdot \mu$$

where μ = electrophoretic mobility in $cm^2\ sec^{-1}\ volt^{-1}$

η = viscosity in poises

D = dielectric constant (dimensionless).

The potential difference across the air/water interface before and after spreading of a monolayer can also be measured (93). The difference is also termed "surface potential (ΔV)". The surface potential of a monolayer depends primarily on the characteristics of the polar groups in the monolayers. Hence, it is a most appropriate parameter for studying ionic interactions in monolayers (94).

As well as binding counterions of salts present in the bulk phase, the charged phospholipid interface will also attract H^+ or OH^- depending on the sign of the charge. This asymmetry of hydrogen or hydroxyl ion distribution means that the surface pH will be different from that measured in the bulk phase. Assuming a Boltzmann distribution and no solvation effects, the pH at the surface will be related to the bulk pH as follows:

$$pH_{surface} = pH_{bulk} + \frac{\varepsilon\psi}{2.3\ kT}$$

where ψ is the surface potential in mv, ε = electronic charge, T = absolute temperature, k = Boltzmann constant.

D. Phospholipases

1. Some Properties of Phospholipases A, B and C: Relationship between the Phospholipase Activity and the Physical Properties of the Substrates

Bangham and Dawson (95-102) have used a variety of anionic and cationic detergents to alter the electrophoretic mobilities of phospholipid dispersions in connection with the role of such dispersions as substrates for phospholipases. They have shown that the

rates of reaction are appreciably influenced by the electrokinetic properties of the enzymes and their substrates. The activity of phospholipase B of Penicillium notatum requires a negative ζ-potential at the substrate surface before hydrolysis commences (96-97). Phospholipase C from Clostridium perfringens requires a negatively charged enzyme and excess positive groups on the substrate (98-99). Triphosphoinositide phosphomonoesterase from brain requires that the negative ζ-potential of the substrate be considerably reduced before hydrolysis can commence at an appreciable rate (100). Dawson (101-102) has investigated the action of the purified phospholipase A_2 from Naja naja on lecithin and phosphatidylethanolamine in relation to the electrophoretic mobility of the lipid particles. In contrast to the above, he found that the activity of cobra-venom (Naja naja) phospholipase A_2 was essentially independent of the ζ-potential of the substrate.

From studies of the mechanism of diethyl ether activation of lecithin hydrolysis by snake venom phospholipase A_2, Dawson (101-102) concluded that penetration of the solvent into the lipid particles causes a wider spacing of the substrate molecules. This was assumed to improve the access of the enzyme to the ester linkage and to prevent the inhibition of the enzymic hydrolysis by removing the fatty acid formed.

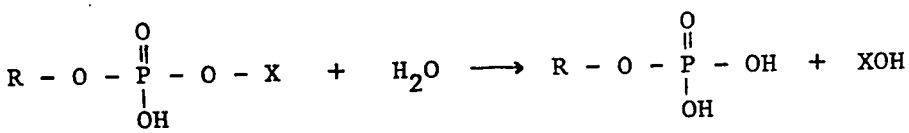
Hughes (103) showed that compressed monolayers of lecithin were not hydrolyzed by snake venom phospholipase A_2. Bangham and Dawson (97) have studied this effect using P^{32}-labeled lecithin spread as monolayers at different pressures. They found that the

phospholipase B of Penicillium notatum attacked low pressure films (< 30 dynes cm^{-1}) and that the rate of hydrolysis decreased until a critical pressure was reached above which hydrolysis no longer occurred. This result could be interpreted as meaning that loose packing of the monolayer allowed the active center of the enzyme to reach the site for hydrolysis.

Many phospholipases which hardly attack coarse lecithin particles can hydrolyze ultrasonically dispersed lecithins at an appreciable rate (104-106). This could be due in part to the increase in surface area available for enzyme attack. Sonication of egg lecithin gives a high resolution NMR spectrum (107). This is probably due not only to reduction in particle size but also to changes in molecular arrangement (108).

2. General Properties of Phospholipase D

The enzyme phospholipase D (EC 3.1.4.4) which occurs in several plants and in Corynebacteria, has been shown to catalyze the following reaction (109-118):



where R is 1,2-diacyl-sn-glycerol or a monoacyl-sn-glycerol and X is choline, ethanolamine, serine or glycerol. In addition to this phosphatidohydrolase activity, the enzyme also exhibits transphosphatidylase activity in the presence of high concentrations of primary alcohols (119-122).

An insoluble form of this enzyme has been isolated from carrot root, sugar beet, spinach and cabbage leaves by Kates (109-111) and does not require Ca^{+2} for its activity. However, a soluble form which requires Ca^{+2} for activity has been isolated from cabbage (112, 123), carrot (115), peanut (122) and cottonseed (124). Recently, a new enzyme belonging to the group phospholipase D has been found in Corynebacterium ovis (125-126) which does not require Ca^{+2} for its activity. Antia, Bilinski and Lau (127) also demonstrated the presence of a phospholipase D in the unicellular red alga, Porphyridium cruentum.

The activity of the cabbage leaf enzyme can be stimulated by diethyl ether (106, 109, 112), by other organic solvents, especially linear aliphatic ketones and esters (128), by acidic phospholipids such as phosphatidic acids and triphosphoinositides (106), by monocetylphosphoric acid (106) and by anionic detergents such as sodium dodecyl sulfate (106, 128). The enzyme is inhibited by cationic detergents such as cetyltrimethylammonium bromide (106) and cetylpyridinium chloride (128), by choline and ethanolamine (106), by EDTA (106, 112), by protamine sulfate (106) and by fluoride ions (109). It is completely inhibited in 0.1 mM p-chloromercuribenzoate (119).

(a) Substrate Specificity

Phospholipase D shows a broad specificity towards phospholipids. It is active toward phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in decreasing order of hydrolysis rate (111-112). It also acts on lysolecithin (112-113) and phosphatidylglycerol (114). Although it acts readily upon the phospholipids possessing the L-α-structure (100-112), it attacks substrates with the DL-α or

the β-structure at much slower rates (112). Thus the enzyme is not strictly stereospecific. When diethyl ether is used as an activator, both saturated and unsaturated substrates can be hydrolyzed by the enzyme. However, the unsaturated compounds are more rapidly hydrolyzed (111-112). Plasmalogens also can be hydrolyzed at a slower rate (41,129).

(b) Optimum pH

When diethyl ether is used as an activator, phospholipase D either in soluble or insoluble form is active in the pH range 4.0 - 6.0. However, the optimal pH varies with the source of the enzyme, being 4.7 for spinach and sugar beet chloroplasts (109) and 5.6 - 5.8 for cabbage and carrot root chloroplasts and for the soluble form of the enzyme in Savoy cabbage (106, 109, 112), carrot root (115) and cottonseed (124). For the ultrasonically irradiated lecithin, the optimal pH for the soluble enzyme is 4.9 (130). When anionic amphipaths are used as activators, the optimal pH shifts to about 6.5 (130).

(c) Stability

The stability of the soluble enzyme is highly dependent on its state of purity. In crude preparations it is stable for 5 minutes at 55°C but it becomes progressively more thermolabile on purification (112). The plastid enzyme is completely inactivated after 10 minutes at 70°C, but is relatively stable between 25 and 40°C (109).

(d) Transphosphatidohydrolase activity

Phospholipase D prepared from cabbage and peanut (119-122) also exhibits transphosphatidohydrolase activity. According to Yang et al. (119) the transferase activity is best accounted for by the following mechanism:

Phosphatidylcholine + HS - enzyme ⟶ phosphatidyl - S - enzyme + choline

Phosphatidyl - S - enzyme + XOH ⟶ phosphatidyl - O - X + HS - enzyme

Phosphatidyl - O - enzyme + H_2O ⟶ phosphatidic acid + HS - enzyme

However, the existence of a phosphatidyl - S - enzyme should be regarded as tentative.

(e) The Activation Mechanism by Organic Solvents and Anionic Amphipaths

Diethyl ether stimulates considerably the hydrolysis of phospholipids by phospholipase D from cabbage, carrot root and peanut (106, 109, 112, 122). However, it does not seem to be required for all phospholipids. For instance, Davidson and Long (112) have indicated that diethyl ether is inhibitory for the hydrolysis of lysolecithin and Heller and Arad (122) have shown that the cleavage of cardiolipins by phospholipase D is inhibited by diethyl ether. In the case of ultrasonically irradiated dispersions of lecithin (105-106) or when the lecithins are dispersed by detergent (106, 109-111), diethyl ether is not required for enzyme activity.

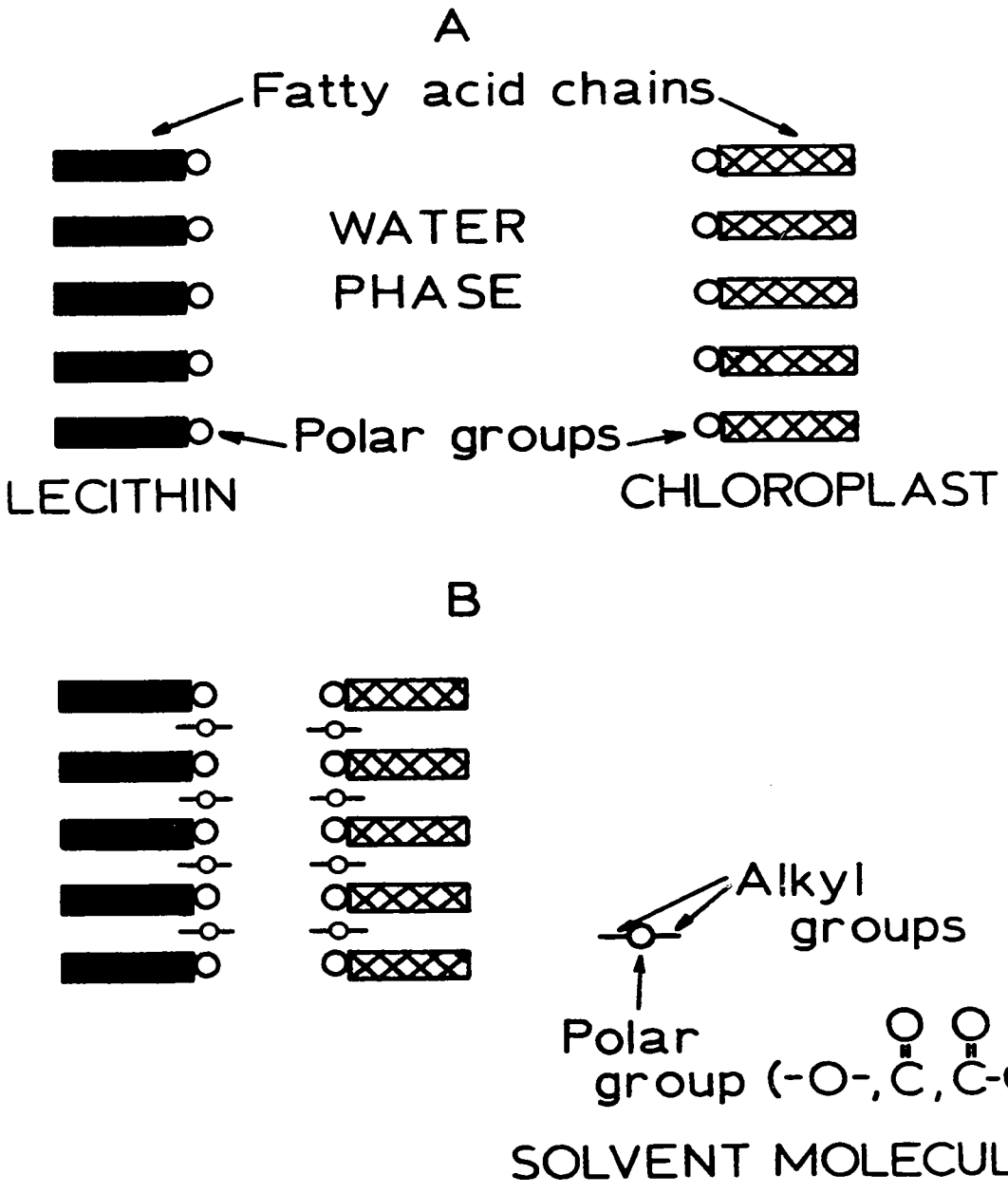
The activating effect of diethyl ether and certain linear aliphatic ketones and esters on the hydrolysis of lecithin has been interpreted by Kates (128) as relating to the physical form of the substrate. In his studies of the insoluble plastid phospholipase D, Kates (128) has shown that all stimulating solvents cause the plastid enzyme and substrate particles to coalesce while non-stimulating solvents such as chloroform did not produce coalescence. It was also shown that the

hydrolytic reaction actually takes place in the coagulum. Kates suggested that the stimulating solvents (128) are adsorbed at the surface of the particles as shown in Fig. 3. On this basis, the polar group in the solvent molecule (ether or carbonyl group) would be attracted to the polar groups at the surface of the particles, whereupon one of the hydrocarbon groups attached to the solvent polar group would penetrate the surface while the other would project outwards, making the surface sufficiently lipophilic for coalescence to occur.

Heller and Arad (122) have indicated that the soluble phospholipase D from peanut catalyzed the hydrolysis of egg lecithin only in the aqueous phase of the biphasic water-diethyl ether system. However, Chen and Barton (131) have shown with soluble phospholipase D from cabbage leaf that the hydrolysis of egg lecithin occurs at the diethyl ether-water interface.

The activating effect of amphipathic compounds on the hydrolysis of lecithin has been described by Kates (128) and by Dawson and Hemington (106). With the plastid enzyme, Kates (128) suggested that the anionic detergents may produce coalescence of substrate and plastid through neutralization of the positive charges of the quaternary nitrogen groups of the choline residues on the surfaces of the particles by the negatively charged anionic detergent micelles. This mechanism would also account for the ineffectiveness of cationic and non-ionic detergents. Hemington and Dawson (106), with soluble phospholipase D, suggested that anionic amphipathic compounds may provide an appropriate negative surface charge density for enzyme binding. They have shown that the addition of sodium dodecyl sulfate promotes the adsorption of the enzyme onto the surface of a lecithin

Figure 3. Mechanism of stimulation of plastid phospholipase D activity by solvents. A, orientation of lecithin molecules at a micelle surface and of phospholipids at the chloroplast surface. B, orientation of linear aliphatic ether, ketone, or ester molecules at lecithin and plastid surfaces (Reference 128).


A
Fatty acid chains
WATER
PHASE
Polar groups
LECITHIN
CHLOROPLAST
B
Alkyl
groups
Polar
group
(-O-, C, C-O)
SOLVENT MOLECULE

substrate (106). This adsorption of the enzyme is probably an essential prerequisite for the formation of an enzyme-substrate complex and may explain the mechanism of the activation by anionic amphipaths. They could not find any evidence that the amphipathic compound produces an increased dispersion of the lecithin. Furthermore, electrophoretic mobility measurements of the mixed lecithin-amphipath micelles in the presence of Ca^{2+} did not show any correlation with the activating effect of amphipaths on the hydrolysis of lecithin.

Quarles and Dawson (130) have shown that the activity of soluble cabbage leaf phospholipase D was much greater in systems in which anionic amphipathic substances were mixed with the lecithin than in systems with diethyl ether as activator or with ultrasonically treated lecithin. They also indicated that amphipathic compounds such as sodium dodecyl sulfate or phosphatidic acid cause a shift of the pH optimum of lecithin hydrolysis from 5.6 to 6.5 (130). Thus, although limited enzyme substrate adsorption probably occurs with pure lecithin, it could be very much greater when the highly negatively charged amphipathic compounds are introduced (106).

The physical state of the phospholipid is of primary importance in the determination of the level of enzymic activity. For instance, the phospholipids present in rat liver microsomes are cleaved by phospholipase D in the absence of diethyl ether (122). Sphingomyelin bound to the microsomes can be hydrolyzed by peanut phospholipase D (122) while pure spingomyelin is not hydrolyzed. It could be reasoned that microsomes exert a stimulating effect through the formation of appropriate forms of substrate for hydrolysis by phospholipase D.

E. Aims of the Work Described in this Thesis

At present, there are two major concepts regarding the mechanism of phospholipid hydrolysis by phospholipases.

1. Electrostatic Effects

Bangham and Dawson (95-102) have attempted to show that various phospholipases will hydrolyze lecithins only when activated with anionic or cationic detergents. The activators were shown to introduce on the surface of the substrate particles a net surplus of charges which were thought necessary for the initiation of the enzyme attack.

2. Dispersive Effects

From studies of the mechanism of the ether activation of lecithin hydrolysis by snake venom phospholipase A_2, Dawson (101-102) concluded that penetration of the ether molecules into the lipid particles causes a wider spacing of the substrate molecules. This was assumed to improve the access of the enzyme to the lipid substrates. Dawson and his associates (104, 130) have also shown that ultrasonic dispersions of egg lecithin can be readily hydrolyzed by phospholipase A_2 of snake venom or by soluble phospholipase D of cabbage leaf in the absence of diethyl ether. Kates (105) also demonstrated that ultrasonically dispersed lecithin can be readily hydrolyzed by the Penicillium notatum phospholipase B in the absence of any activator and that addition of cardiolipin to the ultrasonified lecithin increased the initial rate of hydrolysis only to a relatively small extent. This evidence suggests that the initial attack of phospholipases on the phospholipid particles is dependent on the degree of dispersion of substrate particles rather than on the surface charge of the substrate particles.

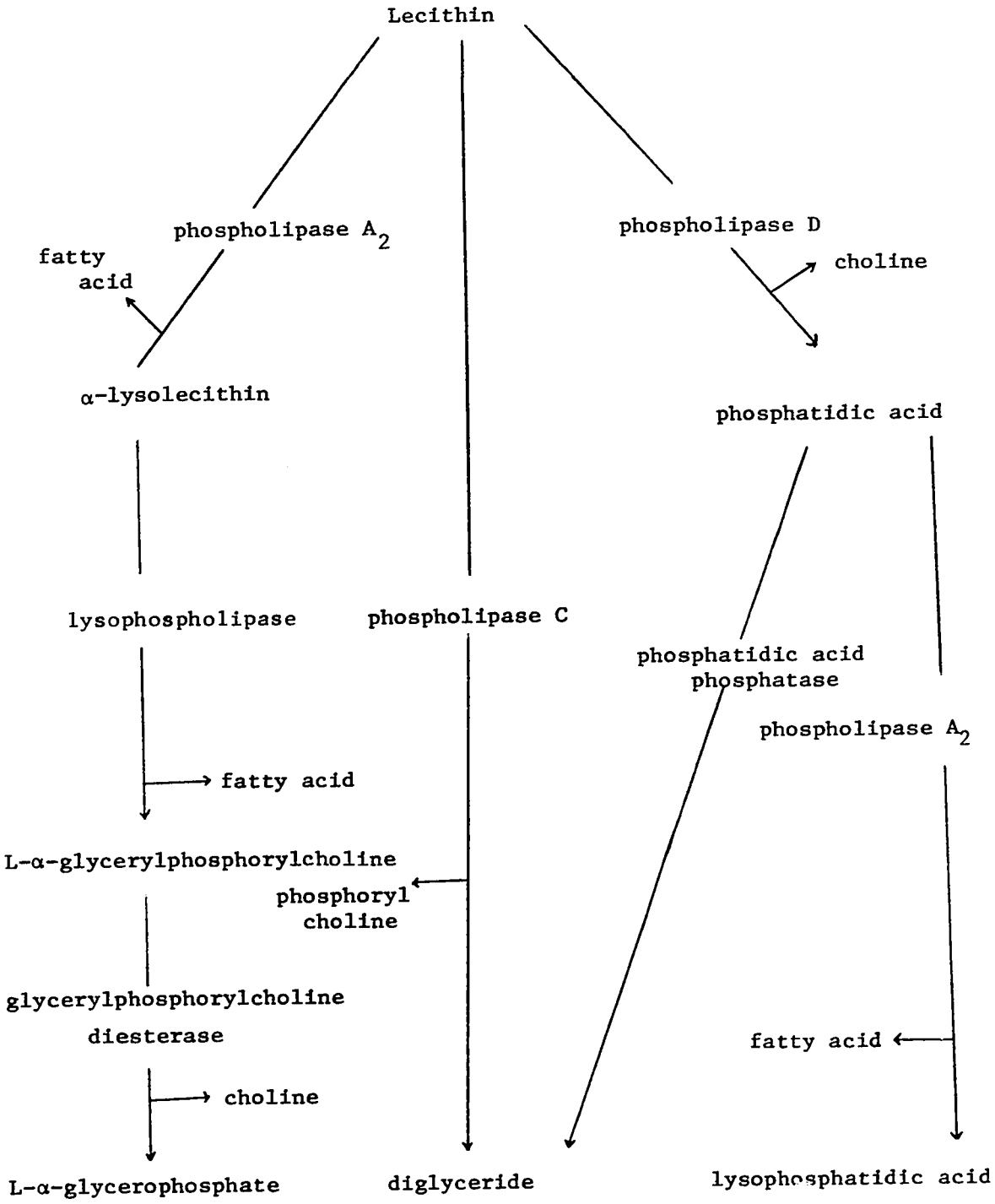
Bangham and Dawson (97) also demonstrated that phospholipase B does attack a monomolecular film of pure lecithin in the absence of anionic amphipaths at low surface pressure (18-26 dynes/cm), but does not attack a high pressure monomolecular film (> 30 dynes/cm). In the latter case, the addition of negatively charged amphipaths allows the enzyme to hydrolyze the phospholipid molecules (97). Whether this is due to the negative charge on the film or to increased spacing of the lecithin molecules remains to be determined.

In the work described in this thesis, an attempt has been made to resolve these two conflicting concepts using the soluble phospholipase D from cabbage leaf as a model.

F. Choice of Substrates

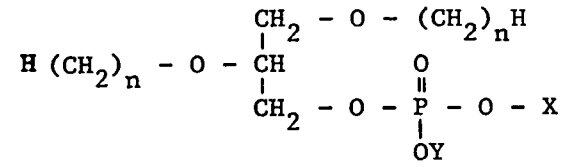
Several routes for the enzymic liberation of choline from lecithin are theoretically possible (see Fig. 4). The direct route utilizes true phospholipase D activity. A second route might proceed through enzymic deacylation followed by phosphodiesterase hydrolysis of the water-soluble L-α-glycerylphosphorylcholine (L-α-GPC) intermediate. Davidson and Long (112) demonstrated the presence of phosphodiesterase activity in cabbage leaf homogenate supernatants which could be separated from phospholipase D by adsorption onto $Ca_3(PO_4)_2$ gel. However, Dawson and Hemington (106) reported that large losses of phospholipase D activity resulted from this procedure and this was confirmed in preliminary experiments in our laboratory. Consequently, an alternative procedure appeared necessary to circumvent the possible formation of intermediate L-α-GPC.

Figure 4. Hydrolytic degradation of lecithin by phospholipases.


Lecithin
phospholipase A2
fatty acid
α-lysolecithin
phospholipase D
choline
phosphatidic acid
lysophospholipase
phospholipase C
phosphatidic acid phosphatase
phospholipase A2
fatty acid
L-α-glycerylphosphorylcholine
phosphoryl choline
glycerylphosphorylcholine diesterase
choline
fatty acid
L-α-glycerophosphate
diglyceride
lysophosphatidic acid

With this in mind, I decided to synthesize by established procedures (30,35) a number of dialkyl ether phospholipids which would not be susceptible to enzymic removal of the hydrocarbon residues for use as model substrates for phospholipase D. The structures of the compounds synthesized are given in Fig. 5. It was anticipated that the availability of these compounds would allow us to explore some facets of the enzymic hydrolysis of phospholipids by phospholipase D not previously amenable to study.

Figure 5. Structures of synthetic ether phospholipids.



when Y = H

$X = -CH_2CH_2\overset{+}{N} \equiv (CH_3)_3$

and n = 1 : 1,2-dimethyl-sn-glycero-3-phosphorylcholine

n = 14: 1,2-ditetradecyl-sn-glycero-3-phosphorylcholine

n = 16: 1,2-dihexadecyl-sn-glycero-3-phosphorylcholine

n = 18: 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine.

when Y = H

X = H

and n = 14: 1,2-ditetradecyl-sn-glycero-3-phosphoric acid

n = 16: 1,2-dihexadecyl-sn-glycero-3-phosphoric acid

n = 18: 1,2-dioctadecyl-sn-glycero-3-phosphoric acid.

when $Y = CH_3$

$X = -CH_2CH_2\overset{+}{N} \equiv (CH_3)_3$

and n = 18: 1,2-dioctadecyl-sn-glycero-3-(methyl)phosphorylcholine.

CHAPTER II

GENERAL MATERIALS AND METHODS

A. Materials

1. Solvents

All solvents were reagent grade unless otherwise specified. Anhydrous pyridine was prepared by distillation over BaO according to Vogel (134) and anhydrous chloroform was prepared according to the same reference.

2. Chemicals

Ag_2CO_3 was a product of Johnson, Matthey and Mallory Ltd., $NaBH_4$ a product of British Drug House, and platinum oxide (Adams' catalyst) a product of Matheson. D-mannitol, lead oxide, sodium periodate, palladium chloride and Fehling's solution A and B were obtained from Fisher. Cetyltrimethylammonium bromide was obtained from Sigma Chemical Company. BRIJ 58 (polyoxyethylene (20) cetyl ether) was obtained from Atlas Chemical Industries. 1-Bromododecane, 1-bromotetradecane, 1-bromohexadecane, 1-bromooctadecane, methyl iodide, monophenylphosphorodichloridate, diphenylphosphorochloridate and choline chloride were products of Eastman Organic Chemicals. Methylphosphorodichloridate was obtained from Frinton Laboratories. Sodium dodecyl sulfate was obtained from Fisher and recrystallized from absolute ethanol. Lead tetracetate was prepared according to Bailar (135). Choline iodide was prepared according to Baer and Kindler (6).

3. Chromatographic Materials

(a) Column Chromatography

Silicic acid (100 mesh) and SilicAR (cc-7, 200-325 mesh) were obtained from Mallinkrodt Chemical Works and were heated at 120°C overnight before use.

Celite 545 was obtained from Fisher.

Aluminum oxide (neutral) A G-7, 100 - 200 mesh, was a product of Bio-Rad Laboratories.

(b) Thin-layer Chromatography

Silica gel G and silica gel H were obtained from Brinkmann Instruments (Canada).

4. Phospholipids

Egg lecithin was isolated from egg yolk as described by Lea, Rhodes and Stoll (137).

Hydrogenated egg lecithin was prepared according to the method of Kates (111).

Phosphatidic acid was prepared from egg lecithin as described by Dawson and Hemington (106) and was converted to the disodium salt (138).

Sphingomyelin (from bovine brain) was obtained from Sigma Chemical Company.

Plasmalogen (choline fraction) was prepared from beef heart according to the method of Warner and Lands (139) and Gottfried and Rapport (140).

1,2-Dioctadecyl-sn-glycero-3-phosphorylcholine was synthesized essentially as described by Stanacev, Baer and Kates (30).

1,2-Dihexadecyl-sn-glycerol was synthesized according to the method of Kates, Chan and Stanacev (22).

1,2-Dihexadecyl-sn-glycero-3-phosphorylcholine, 1,2-ditetradecyl-sn-glycero-3-phosphorylcholine, 1,2-dioctadecyl-sn-glycero-3-phosphoric acid, 1,2-dihexadecyl-sn-glycero-3-phosphoric acid, 1,2-ditetradecyl-sn-glycero-3-phosphoric acid were prepared as described in Chapter III.

5. Phospholipase D

Two preparations were used as indicated. Type I from cabbage leaf was purchased from Sigma Chemical Co. Lot numbers 20C - 2090 and 110C - 1490 were used as indicated. Type II was isolated from Savoy cabbage leaves according to the method of Davidson and Long (to stage III) (112). No differences were observed between the two preparations during the course of this work.

6. Buffer Solution

For experiments carried out below pH 6, 0.1 M sodium acetate-acetic acid buffer was used. For the pH range above 6, equal volumes of 0.25 M acetic acid and 0.25 M 2-amino-2-hydroxy-methylpropane-1:3-diol (Tris) were mixed, the pH was then adjusted to the desired value by the addition of HCl or NaOH, and the buffer solution was diluted so that the final concentrations of acetate and Tris were each 0.1 M (112).

B. Methods

1. Column Chromatography

Neutral lipids were resolved on columns of aluminum oxide and phospholipids were resolved on columns of silicic acid/celite 545 (2:1 w/w). The size of the column used was dependent on the total amount of

lipid to be fractionated, a loading factor of 1 g of lipid to 50 g of aluminum oxide or silicic acid being used. The silicic acid was mixed with celite 545 in the ratio of 2:1 (w/w) respectively in order to increase the flow rate. A slurry made up of this mixture in chloroform was poured into a glass column. The lipid material was normally applied in a minimum volume of solvent and washed on to the column with successive small portions of the same solvent. Chloroform was used for elution of the neutral lipids followed by a $CHCl_3$: CH_3OH mixture to elute the phospholipids. For the aluminum oxide column, hexane and hexane:diethyl ether mixtures were used for elution of neutral lipids.

2. Thin-layer Chromatography

Thin-layer chromatographic methods were used both for resolution of the various neutral lipids and for resolution of the phospholipid classes. The thin-layer chromatogram with applied samples was placed in a solvent tank containing the desired developing solvent system. The plate was removed after the solvent had run to the top of the plate and the excess solvent allowed to vaporize. The plate was then sprayed with iodine dissolved in $CHCl_3$ or with 60% H_2SO_4 and charred in an oven at 110°C.

(a) Neutral Lipids

Neutral lipids were separated on Silica gel G thin-layer plates (250 μ thickness) with developing solvent A (petroleum ether: diethyl ether:glacial acetic acid 85:15:1 v/v/v).

(b) Phospholipids

Phospholipids were separated on Silica gel H plate (250 μ thickness) with developing solvent B ($CHCl_3$:CH_3OH:6N NH_4OH, 60:35:5 v/v/v) or with developing solvent C ($CHCl_3$:CH_3OH:glacial acetic acid:H_2O, 60:35:2:3 v/v/v/v).

3. Analytical Methods

(a) Phosphorus Determination

Total phosphorus was determined by the method of King (141) and total phospholipids in the case of naturally occurring compounds were estimated by multiplying this value by a factor of 25.

(b) Protein Determination

Protein was determined by the method of Lowry et al. (142).

(c) Choline Determination

Choline was determined according to the method of Wheeldon and Collins (143).

(d) Determination of α,β-Unsaturated Ether Content

Vinyl ether was determined according to Gottfried and Rapport (140).

(e) Determination of Aldehyde Content

Aldehyde content was determined according to Rapport and Alonzo (144).

(f) Optical Densities (Absorbances)

These were measured with a Beckman DB-G spectrophotometer.

(g) Optical Rotations

These were measured on pure liquids or solutions, as specified, using a Perkin-Elmer 141 polarimeter.

(h) Melting Points (Uncorrected)

These were determined with a Thomas Hoover capillary melting point apparatus.

(i) Refractive Indices

These were measured with a Bausch and Lomb Abbé 3L refractometer.

(j) Densities

These were determined through the use of a pycnometer.

(k) Infrared Spectra

These were obtained on a Beckman IR 10 (pure liquids or solutions) or on a Perkin-Elmer 621 (NaCl pellets).

(l) NMR Spectra

These were obtained on Varian A-60, A56/60A or HA-100A instruments.

4. Preparation of Lipid Samples for Enzymic Hydrolysis

The phospholipid samples were prepared by dispersing them in buffer solution with a Vortex mixer.

5. Differential Thermal Analysis (DTA)

Differential thermal analysis was carried out with a Du Pont 900 Differential Thermal Analyzer. The sample container was a glass capillary tube of 2 mm or 4 mm diameter. The rate of heating was approximately 8°C/minute. For the cooling run, cooled nitrogen was circulated. Thermograms were recorded on chart paper corrected for the non-linearity of the alumel-chromel thermocouples.

6. DTA Sample Preparation

Mixtures of phospholipids and water or SDS and water were prepared by sealing weighed amounts of the components into glass capillary tubes and repeatedly centrifuging the material from one end of the tube to the other at a temperature above that estimated to be required for liquid-crystal formation. If two or more lipid components were used, they were first dissolved in $CHCl_3$, the solvent was removed with a stream of nitrogen, and the samples were then dried *in vacuo*.

7. Evaporation

All evaporations were carried out under reduced pressure (water pump) at 30-35°C on a water bath using a Calab Model C evaporator.

8. Hydrogenation and Hydrogenolysis

Hydrogenation and hydrogenolysis reactions were carried out in a Parr Shaker Apparatus, Series 39110 (250 ml bottle).

9. Enzymic Assay System

The enzymic reaction was normally carried out in 12 ml graduated centrifuge tubes.

(a) Standard Reaction Mixture A

When an organic solvent was used as activator, the reaction system was essentially that described by Davidson and Long (112). A standard reaction mixture (A) contained 16 µmoles phospholipids, 1.25 ml of 0.1 M acetate buffer, pH 5.6, 0.25 ml of 1 M $CaCl_2$, 1 ml of enzyme (1-4mg protein/ml) solution and 1ml of organic solvent and was incubated at 27°C. A second system containing half-quantities was used as specified.

(b) Standard Reaction Mixture B

When phosphatidic acid was used as activator, an appropriate amount of phosphatidic acid in $CHCl_3$ was mixed with a $CHCl_3$ solution of the substrate and the solvent was removed with a stream of N_2 and dried in vacuo. When SDS was used as activator, an appropriate amount of SDS solution (80 μmoles/ml) was added to the dried phospholipids. The mixtures were then dispersed in 1.25 ml of 0.1 M buffer solution and 0.25 ml of 1 M $CaCl_2$ was added and mixed. 1.0 ml of enzyme solution was added and the contents of the tube were mixed and incubated for an appropriate time at the temperature indicated.

After incubation, the enzymic reaction was stopped by addition of 1 ml of 30% (w/v) trichloracetic acid, 1.5 ml of water was added, the mixture shaken vigorously and the tube centrifuged. The aqueous phase was filtered and 1 ml was used for choline determination. If organic solvent had been added, the filtrate was warmed to 55°C in a stream of nitrogen to remove the dissolved solvent.

With substrates which were sparingly soluble in diethyl ether, 0.1 ml of $CHCl_3$ was added to the reaction mixture as well as 1 ml of ether (112).

CHAPTER III

CHEMICAL SYNTHESIS OF DIALKYL ETHER PHOSPHOLIPIDS

A. Synthesis of Intermediate Compounds

1. 1,2,5,6 Diisopropylidene-D-Mannitol (Diacetone-D-Mannitol)

Diacetone-D-mannitol was prepared from D-mannitol by a modification (145-146) of the original method of Baer and Fischer (147).

2. 1,2-Isopropylidene-sn-Glycerol (D-Acetone Glycerol)

1,2-Isopropylidene-sn-glycerol was prepared from 1,2,5,6-diisopropylidene-D-mannitol by the methods of Baer and Fischer (147), modified through the use of sodium borohydride as reducing agent (148-149).

3. 3-Benzyl-sn-Glycerol (L-α-Benzyl Glycerol)

3-Benzyl-sn-glycerol was prepared from 1,2-isopropylidene-sn-glycerol via 1,2-isopropylidene-3-benzyl-sn-glycerol (150-151).

4. 1,2-Dioctadecyl-3-Benzyl-sn-Glycerol (D-α-β-Di-O-Octadecyl-α' Benzyl Glycerol)

This compound was prepared by reaction of 3.65 g (0.02 moles) 3-benzyl glycerol with 26.7 g (0.08 moles) 1-bromo-octadecane and 4.5 g powdered KOH in 50 ml anhydrous benzene, exactly as described by Kates, Chan and Stanacev (22). However, the work-up of the reaction mixture was by a quite different method yielding a purer product.

The crude reaction mixture was diluted with 50 ml anhydrous diethyl ether and washed successively with 200 ml each of water, hydrochloric acid, 2.5% K_2CO_3 solution and water (22). The washed benzene

layer was dried over anhydrous Na_2SO_4, evaporated to dryness under reduced pressure and the residue dissolved in 100 ml n-hexane. The hexane solution was then stirred with several small portions (∿10 g) of alumina until the supernatant was devoid of material having R_F 0.75 on Silica Gel G with solvent A. The pooled adsorbent was poured into a glass chromatography tube (diameter 5 cm) already containing alumina packed in hexane to a height of 10 cm and washed with 900 ml n-hexane which eluted 1-bromo-octadecane. 1,2-dioctadecyl-3-benzyl-sn-glycerol was then eluted with 450 ml 1% diethyl ether in n-hexane followed by 300 ml 4% diethyl ether in hexane. The last four fractions were pooled and the solvent removed by evaporation. The yield of 1,2-dioctadecyl-3-benzyl-sn-glycerol was 11 g (80% theory).

m.p. 38-39°, $[\alpha]_D^{25}$ = -0.35°, (c = 7.5% w/v in chloroform). Infrared absorption spectrum (CCl_4) (Fig. 6). Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): CH_3-C-, 9.14, (2x3H); -CH_2-C-, 8.72; RCH_2-O-CH_2, RCH_2-O-CH, R-O-CH_2-CH, R-O-CH-CH_2, overlapping resonances 6.0 - 6.9, (11H); Ar-CH_2-O-C, 5.44, (2H); C_6H_5-C-, 2.66, (5H) (Fig. 7). Anal. Calcd. for $C_{46}H_{80}O_3$ (687): C, 80.40; H, 12.62. Found: C, 80.85; H, 12.59, 12.25 (5).

5. 1,2-Dioctadecyl-sn-Glycerol (D-α,β-Di-O-octadecyl Glycerol)

1,2-Dioctadecyl-sn-glycerol was obtained by the method of Kates, Chan, and Stanacev (22).

m.p. = 56 - 57.5°, $[\alpha]_D^{25}$ = -7.5° (c = 3.75% w/v in $CHCl_3$).

Reported (22): m.p. = 53.5 - 54.5°·$[\alpha]_D^{21}$ = -6.85° (c = 7.5% w/v in $CHCl_3$). Thin-layer chromatography on Silica Gel G with solvent A gave R_F = 0.35. Infrared absorption spectrum (CCl_4) (cm^{-1}) identical

Figure 6. Infrared absorption spectrum of 1,2-dioctadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the CCl_4 solution using an IR 10 spectrophotometer.

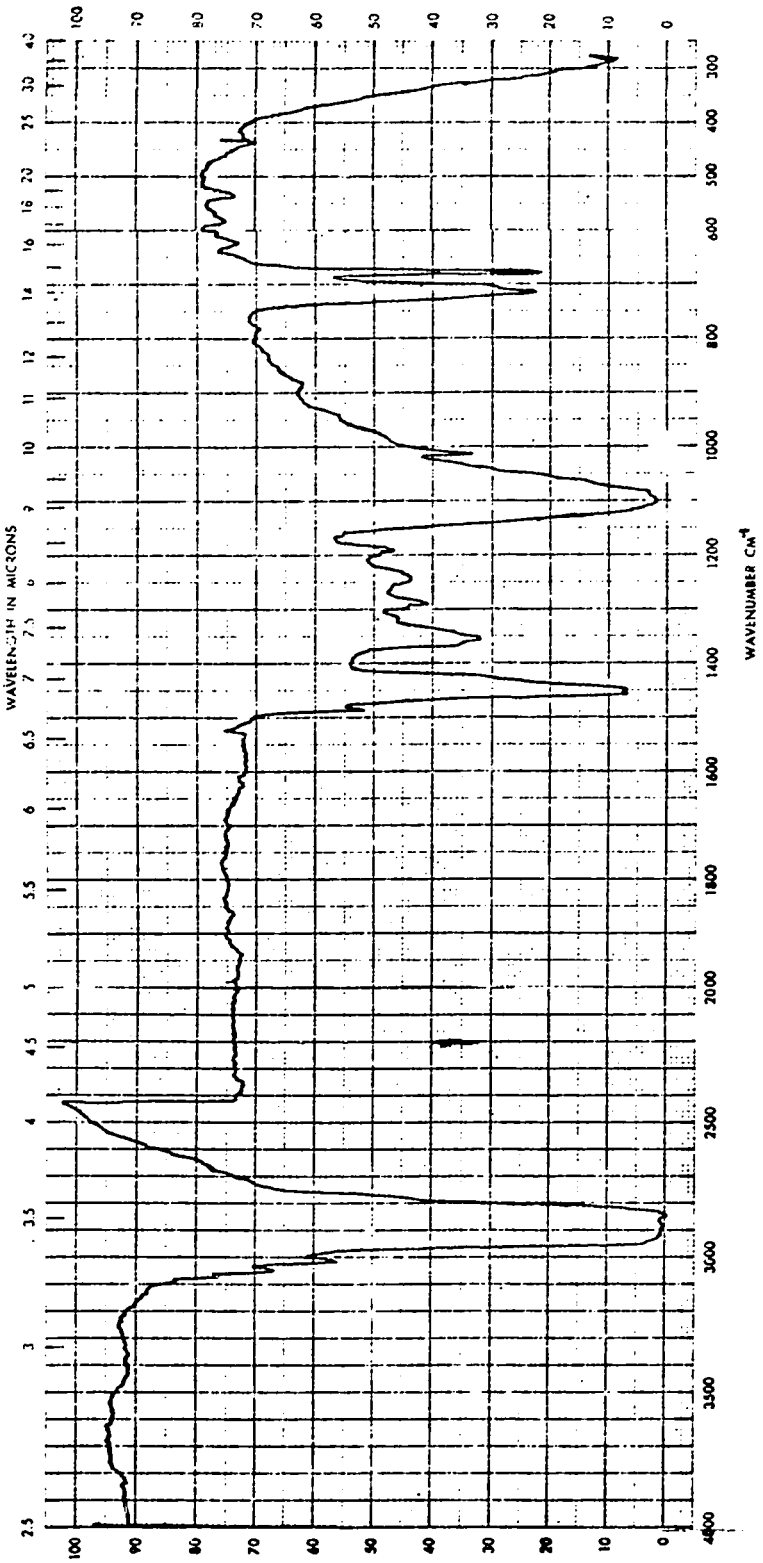
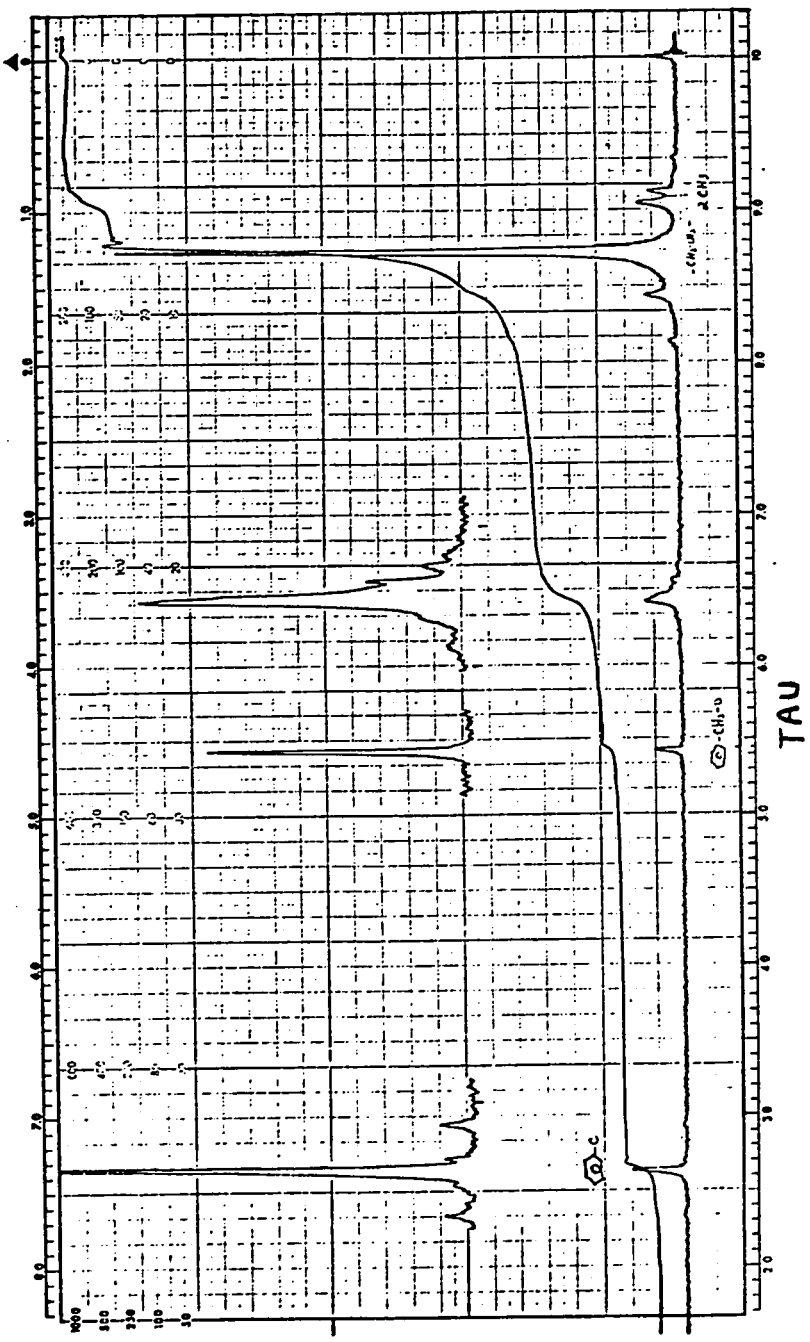

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 7. Nuclear magnetic resonance spectrum of 1,2-dioctadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


TAU

with that reported (22) except that no -OH band was visible at the lower concentration used. Infrared absorption spectrum (Nujol) (cm^{-1}):-OH, 3490 (s, sharp); C-O-C, 1070 - 1095 (s. doublet) (Fig. 8). Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): CH_3-C-, 9.13, (2x3H); $-CH_2$-C-, 8.72, (64H); -OH, 7.80, (1H); RCH_2-O-CH_2-, RCH_2O-CH-, R-O-CH_2-CH, R-O-CH-CH_2-, 6.28 - 6.68, (9H) (Fig. 9). Anal. Calcd. for $C_{39}H_{80}O_3$ (596): C, 78.45; H, 13.51. Found C, 78.84; H, 13.12. Reported (22): C, 78.60; H, 13.84.

6. 1,2-Ditetradecyl-3-Benzyl-sn -Glycerol

The synthesis of this compound was based on the same general principles as that described for the preparation of 1,2-dioctadecyl-3-benzyl-sn-glycerol (22). The compound was prepared by reaction of 20.76 g (0.12 mole) 3-benzyl glycerol with 133.1 g (0.48 mole) of 1-bromo-tetradecane and 27 g of powdered KOH in 300 ml anhydrous benzene. The mixture was refluxed with stirring for 48 hours, the water formed being removed into a phase-separating head. The cooled mixture was diluted with 300 ml of diethyl ether and washed successively with water, 1 N HCl, 2.5% $KHCO_3$ and finally with water. The benzene solution was dried over Na_2SO_4 overnight and the solvent was evaporated under reduced pressure. The residue was subjected to vacuum distillation to remove unreacted 1-bromotetradecane boiling between 114-133°C/0.5 mm Hg (oil bath 160-190°C). The residual oil was further purified as described for 1,2-dioctadecyl-3-benzyl-sn-glycerol. The yield was 38.5 g (60% theory). $[\alpha]_D^{25}$ - 0.14 (pure liquid).

Figure 8. Infrared absorption spectrum of 1,2-dioctadecyl-sn-glycerol. The spectrum was taken with Nujol using an IR 10 spectrophotometer.

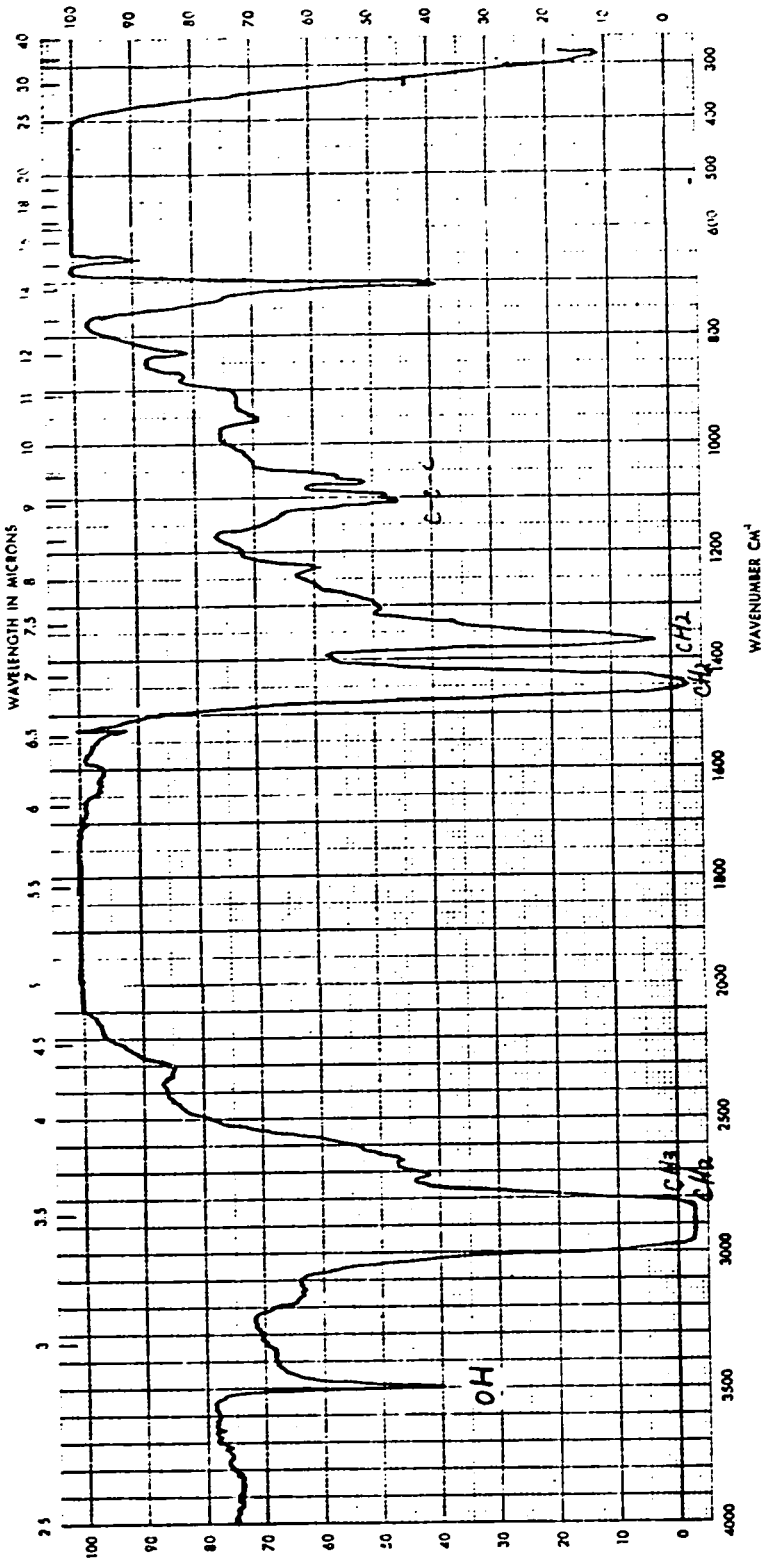
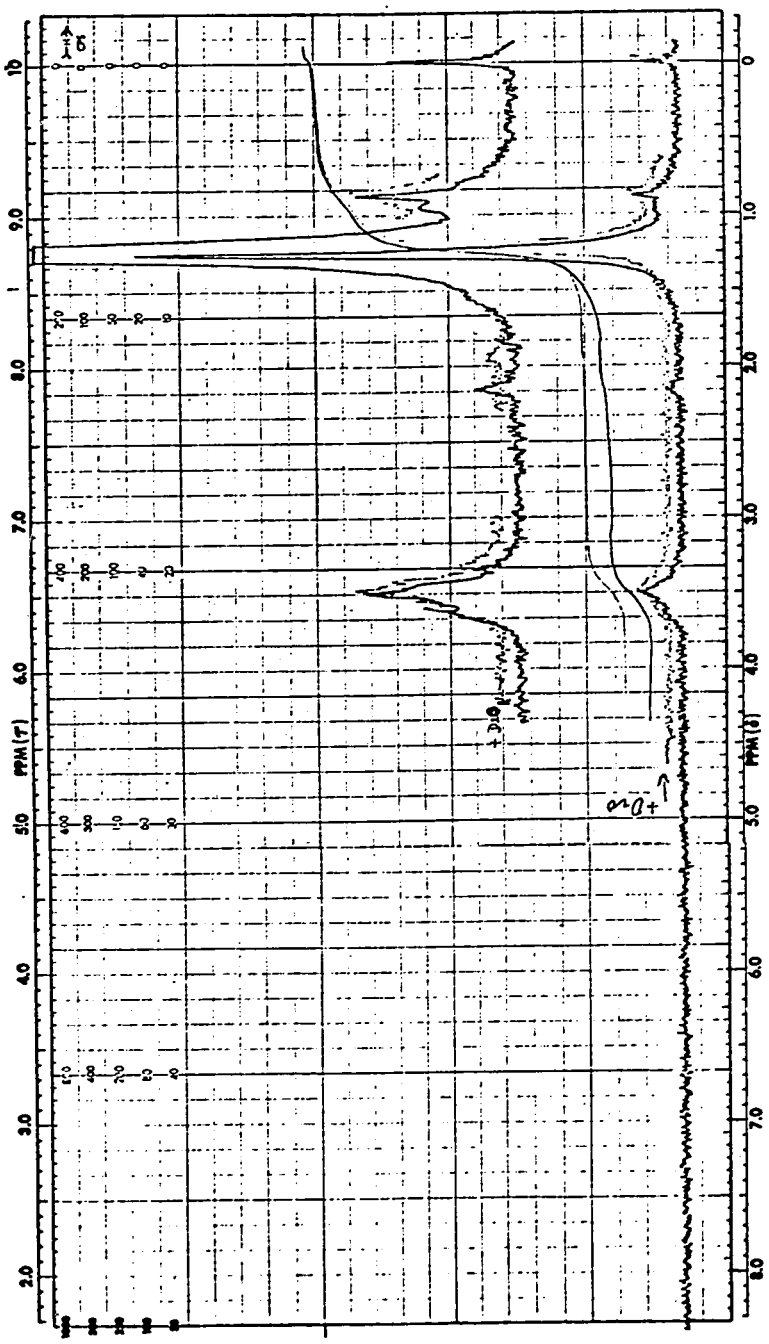

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹
OH
CH₂
CH₃
CH₂
C-C

Figure 9. Nuclear magnetic resonance spectrum of 1,2-dioctadecyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


PPM (τ)
PPM (δ)
10
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
δ
τ

Thin layer chromatography on Silica Gel G with solvent A gave R_F=0.85.

Infrared absorption spectrum (CCl_4) (cm^{-1}): C_6H_5-, 3080 (w), 3050 (w), 3020 (w), 1490 (w); CH_3-, CH_2-, CH-, 2920 (s), 2850 (s), 1465 (s), 1365 (m); C-O-C, 1100 (s). Other bands, 720 (m), 680 (m).

Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): : CH_3-C-, 9.14 (2 x 3H); -CH_2-C-, 8.72 (48H); RCH_2-O-CH_2, RCH_2-O-CH, R-O-CH_2-CH, R-O-CH-CH_2, overlapping resonance 6.0 - 6.9 (9H); Ar-CH_2-O-C, 5.44 (2H); C_6H_5-C, 2.66 (5H).

Anal. Calcd. for $C_{38}H_{70}O_3$ (574.94): C, 79.38; H, 12.27. Found: C, 79.15; H, 12.09.

7. 1,2-Ditetradecyl-sn-glycerol

1,2-ditetradecyl-3-benzyl-sn-glycerol (36 g) in 120 ml of ethyl acetate was maintained at a pressure of 25 p.s.i. hydrogen at room temperature in the presence of 2.5 g freshly prepared palladium on charcoal catalyst (152). The reaction was complete in 1 hour. The catalyst was filtered off and washed with chloroform. The combined filtrate and washings were evaporated to dryness under reduced pressure. The residue was recrystallized several times from 75 ml of ethyl acetate at 4°C. The yield was 25.3 g (80% theory): m.p. 42.5 - 43.5°C. $[\alpha]_D^{25}$ - 8.0 (c 9.3% w/v in $CHCl_3$).

Thin layer chromatography on Silica gel G with solvent A gave an R_F of 0.30.

Infrared absorption spectrum (CCl_4) (cm^{-1}) was identical with 1,2-dioctadecyl-sn-glycerol.

Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): CH_3-C-, 9.14 (2 x 3H); -CH_2-C-, 8.72 (48H); -OH, 7.8 (1H); RCH_2-O-CH_2-, RCH_2-O-CH-, R-O-CH_2-CH, R-O-CH-CH_2-, 6.0 - 6.9 (9H).

Anal. Calcd. for $C_{31}H_{64}O_3$ (484.82): C, 76.80; H, 13.31. Found: C, 77.04; H, 12.93.

8. 1,2-Dimethyl-3-Benzyl-sn-Glycerol (D-α,β-Di-O-Methyl-α' Benzyl Glycerol)

A mixture of 12.81 g (0.07 moles) 3-benzyl-sn-glycerol, 40.32 g (0.28 moles) methyl iodide and 16.1 g (0.28 moles) powdered KOH in 200 ml anhydrous benzene was refluxed with stirring (magnetic stirrer) for 20 hours, the water formed being removed into a phase-separating head. The reaction mixture was brought to room temperature and diluted with 200 ml diethyl ether. The solid material present was stirred with a glass rod and then allowed to settle for one hour. The supernatant was decanted and the insoluble residue washed with two portions of 150 ml anhydrous diethyl ether. The supernatant and washings were combined and the solvent removed by evaporation. The residual oil was subjected to vacuum distillation on an oil bath (140-150°) and the fraction distilling at 112-113° under 0.7 mm Hg pressure collected. The yield was 12.7 g (90% theory).
$d_4^{27.8} = 1.0208$, $n_D^{24} = 1.4901$. $\alpha_D^{25} = +5.17°$, $[\alpha]_D^{25} = +5.06$ (pure liquid). Thin layer chromatography on Silica Gel G with solvent A gave $R_F = 0.33$. Infrared absorption spectrum (pure liquid) (cm^{-1}): C_6H_5-, 3015 (w), 1490 (w); CH_3-, CH_2-, CH-, 2990 (sh), 2910 (sh) 2880 (s), 2820 (sh), 1450 (m), 1350 (m) 1100 (s, broad); other bands, 1300 (w), 1245 (w), 1180 (m), 720 (s), 680 (s).

Nuclear magnetic resonance spectrum: (Fig. 10).

Anal. Calcd. for $C_{12}H_{18}O_3$ (213): C, 68.53; H, 8.62.

Found: C, 68.86; H, 8.39.

9. 1,2-Dimethyl-sn-Glycerol (D-α,β-Di-O-Methyl Glycerol

12.5 g 1,2-dimethyl-3-benzyl-sn-glycerol (0.105 moles) in 50 ml ethyl acetate was maintained at a pressure of 25 p.s.i. hydrogen at room temperature in the presence of 1.25 g freshly prepared palladium on charcoal catalyst (152). The reaction was completed in 15 minutes. The catalyst was filtered off and washed with chloroform. The combined filtrate and washings were evaporated under reduced pressure at 30 - 35° and the liquid residue subjected to vacuum distillation on an oil bath at 80 - 90°. The fraction distilling at 49 - 50° under 0.4 mm Hg pressure was collected. The yield was 6.3 g (80% theory). $d_4^{24} = 1.0048$, $n_D^{26} = 1.4220$, $n_D^{24} = 1.4233$, $\alpha_D^{25} = -5.3°$, $[\alpha]_D^{25} = -5.27°$ (pure liquid). Thin layer chromatography on Silica Gel G with solvent A gave $R_F = 0.18$. Infrared absorption spectrum (pure liquid) (cm^{-1}): -OH, 3400 - 3440 (s, broad); CH_3-, $-CH_2$-, -CH-, 2970 (sh), 2910 (s), 2870 (s), 2810 (sh), 1440 (m), 1390 (w), 1345 (w); C-O-C, 1085 - 1075 (s, broad); other bands, 1620 (w), 1175 (m, sharp), 940 (w), 800 (m) (Fig. 11). Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): -OH, 7.11; CH_3-O-, 6.66, 6.56; $-CH_2$-O, 6.58, 6.51, 6.40, 6.33, -CH-O, 6.3 - 6.7 (multiplet) (Fig. 12). Anal. Calcd. for $C_5H_{12}O_3$ (120): C, 49.98; H, 10.06; Found: C, 49.51; H, 10.19.

Figure 10. Nuclear magnetic resonance spectrum of 1,2-dimethyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.

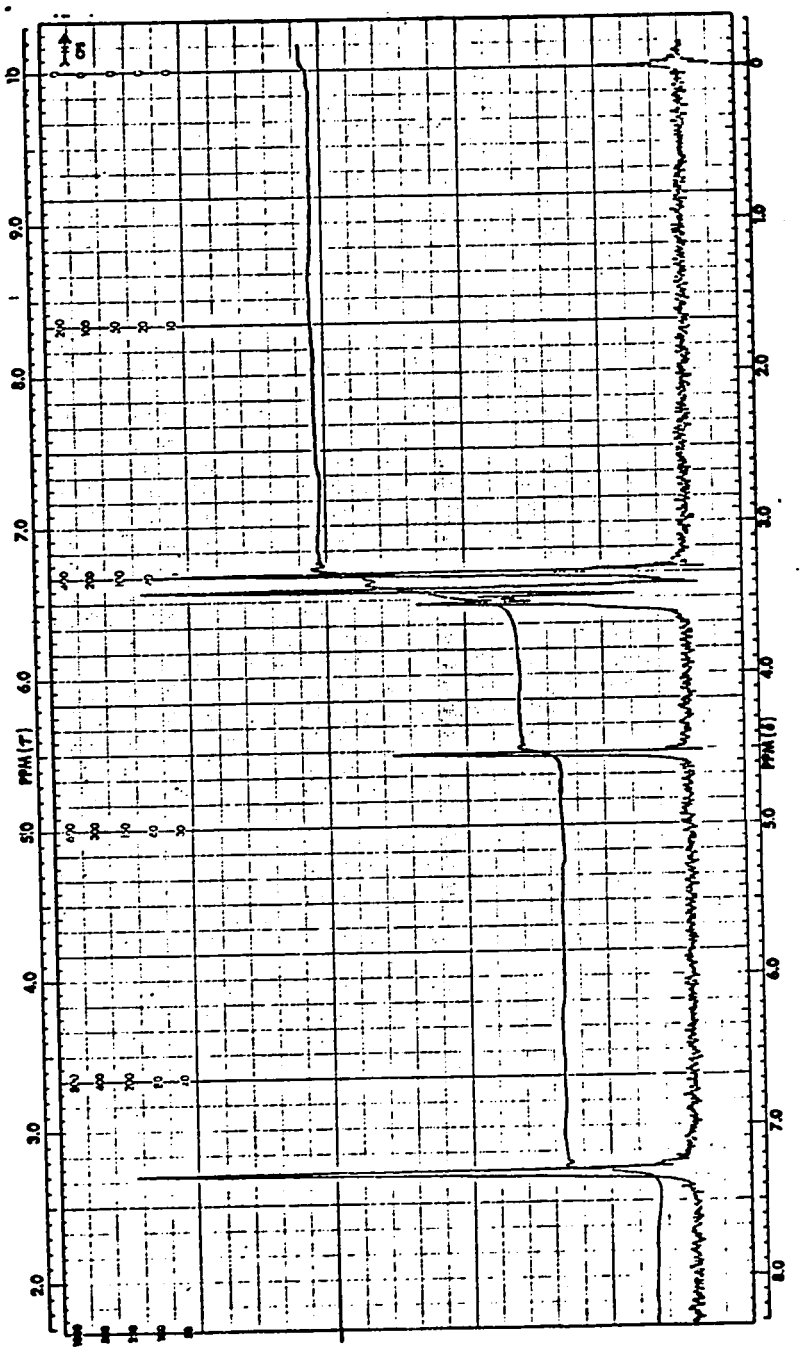

PPM (F1)
PPM (F2)

Figure 11. Infrared absorption spectrum of 1,2-dimethyl-sn-glycerol. The spectrum was taken as the pure liquid using an IR 10 spectrophotometer.

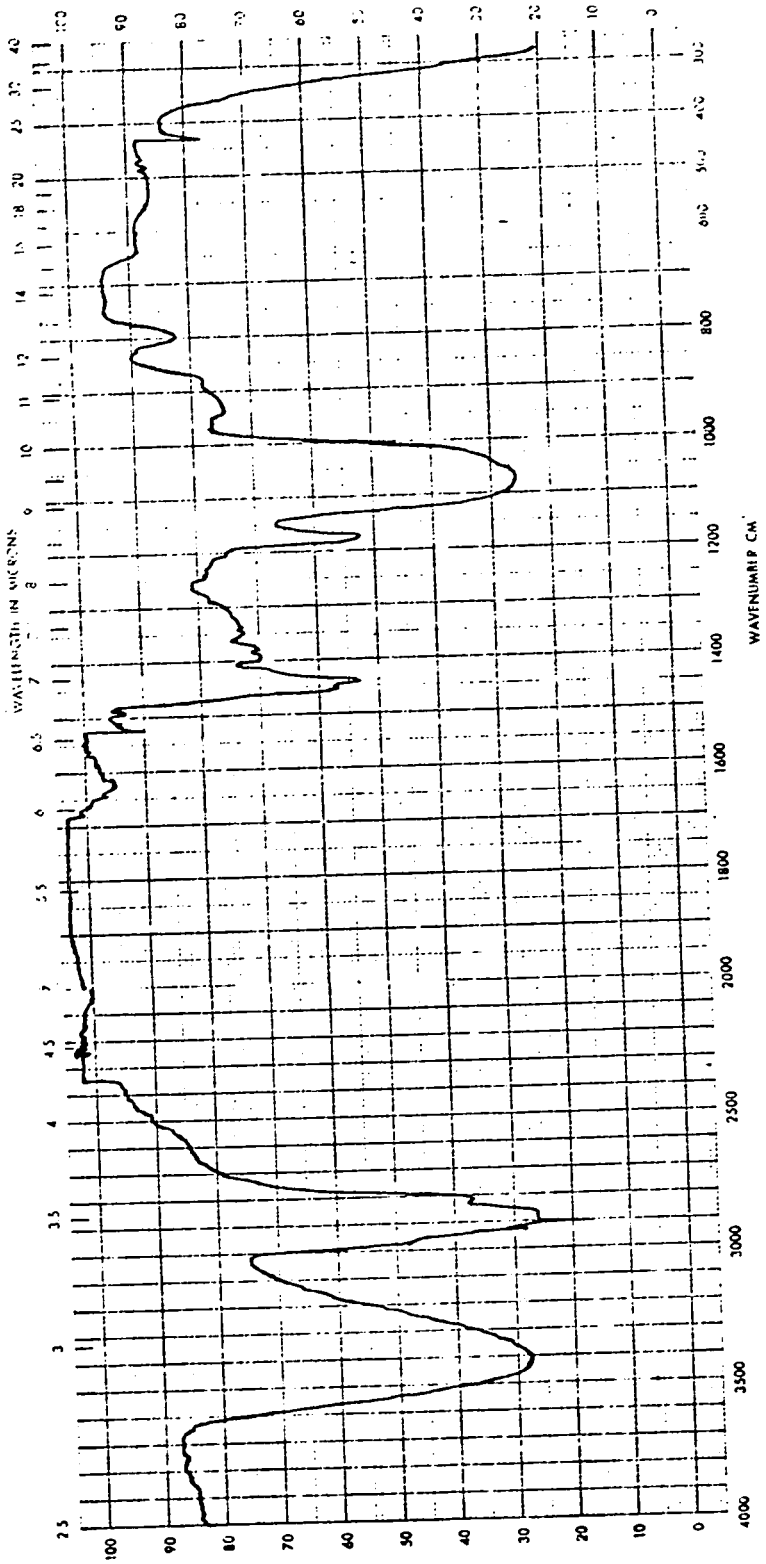
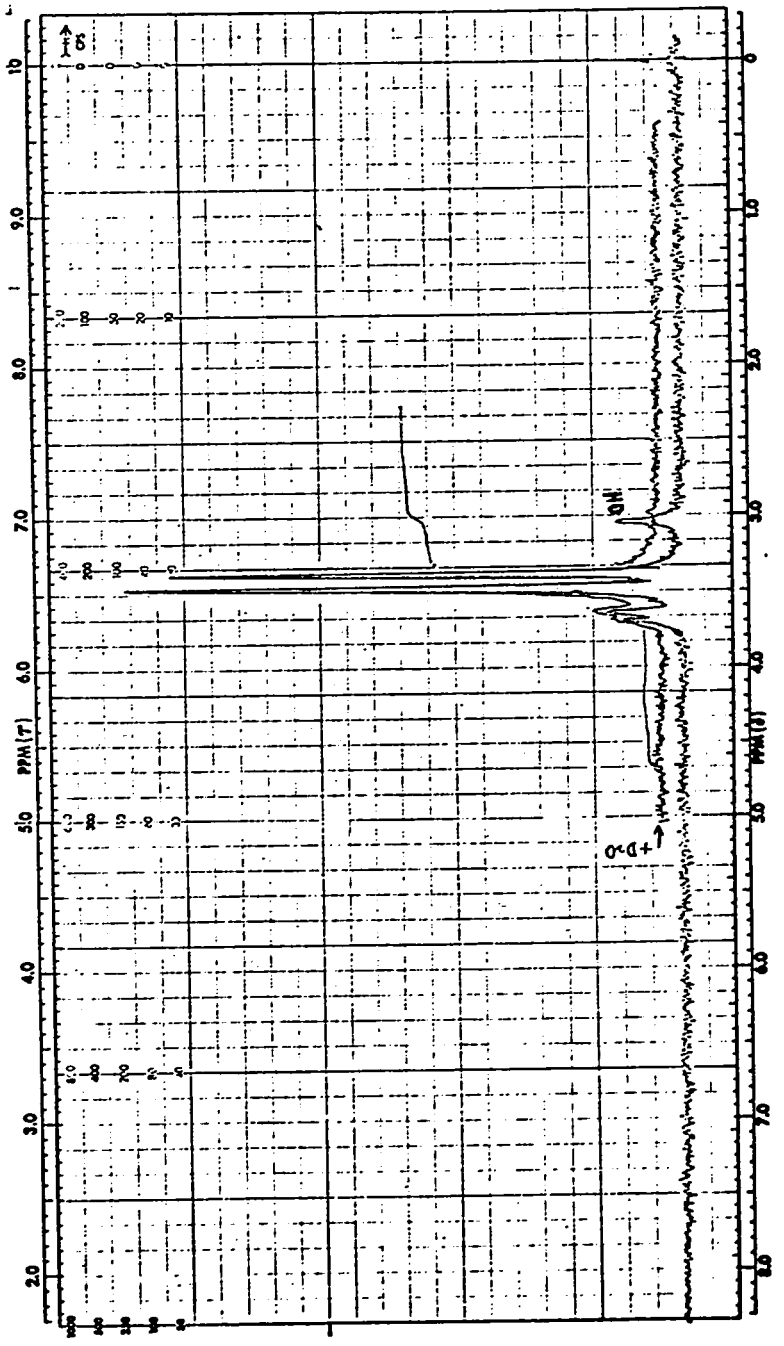

WAVENUMBER CM⁻¹
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
100
90
80
70
60
50
40
30
20
10
0

Figure 12. Nuclear magnetic resonance spectrum of 1,2-dimethyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


δ
PPM(T)
PPM(T)

B. Synthesis of 1,2-Dialkyl-sn-Glycero-3-Phosphoric Acids (Scheme 7)

1. 1,2-Dioctadecyl-sn-Glycerol-3-Diphenyl Phosphoric Acid (Di-O-Octadecyl-L-α-Glycerophosphoric Acid Diphenyl Ester)

The synthesis was based on that used to obtain the diacyl analog (13). 5.96 g (0.01 moles) 1,2-dioctadecyl-sn-glycerol in 62.5 ml anhydrous pyridine was mixed with a solution containing 2.96 g (0.011 moles) diphenylphosphorochloridate in 15 ml anhydrous pyridine. The reaction mixture was maintained under anhydrous conditions at 30° for 19 - 20 hours. Residual diphenylphosphorochloridate was removed by addition of 1 ml distilled water. 65 g chopped ice was added in small portions to precipitate the crude product. Precipitation was completed by adding 400 ml ice-cold distilled water and allowing the mixture to stand for 3 hours at 4°. The precipitate was collected by filtration, washed with water and dried in vacuo over P_2O_5 for four days at room temperature. The residue was extracted into 185 ml warm petroleum ether (b.p. 37.6 - 50.9°), one gram of celite was added and the insoluble material removed by filtration. The filtrate was evaporated to dryness under reduced pressure at 30 - 35° and stored under vacuum. The yield was 7.5 g (90.7% theory).

m.p. 36 - 37° $[\alpha]_D^{25}$ = -2.6° (c = 5.2% w/v in chloroform).

Thin layer chromatography on Silica Gel G with solvent A gave R_F = 0.43. Infrared absorption spectrum ($CHCl_3$) (cm^{-1}):C_6H_5-, 3020 (s, sharp), 1580 (s, sharp), 1470 (s, sharp); CH_3-, -CH_2-, -CH-, 2910 (s), 2850 (s). 1455 - 1460 (s, doublet), 1370 (w); P = O, 1280 (s); P-O-C_6H_5, 1205 (s); C-O-C, 1105; P-O-C, 1185 (s), 1040 (m); other bands 940 (s), 740 (s) (Fig. 13). Nuclear magnetic resonance ($CDCl_3$) (τ): CH_3-C-, 9.12,

Figure 13. Infrared absorption spectrum of 1,2-dioctadecyl-sn-glycero-3-diphenyl phosphoric acid. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophotometer.

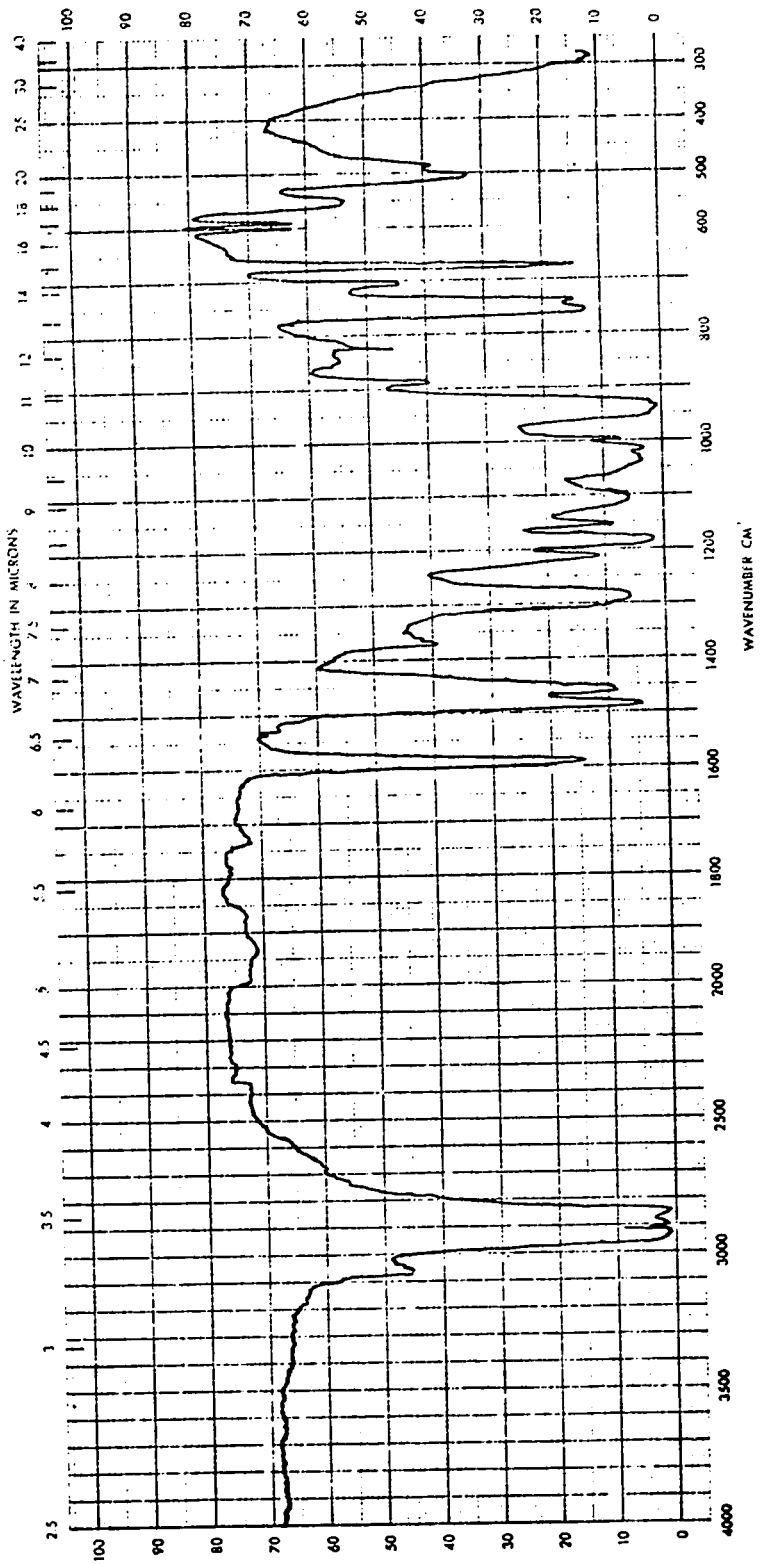
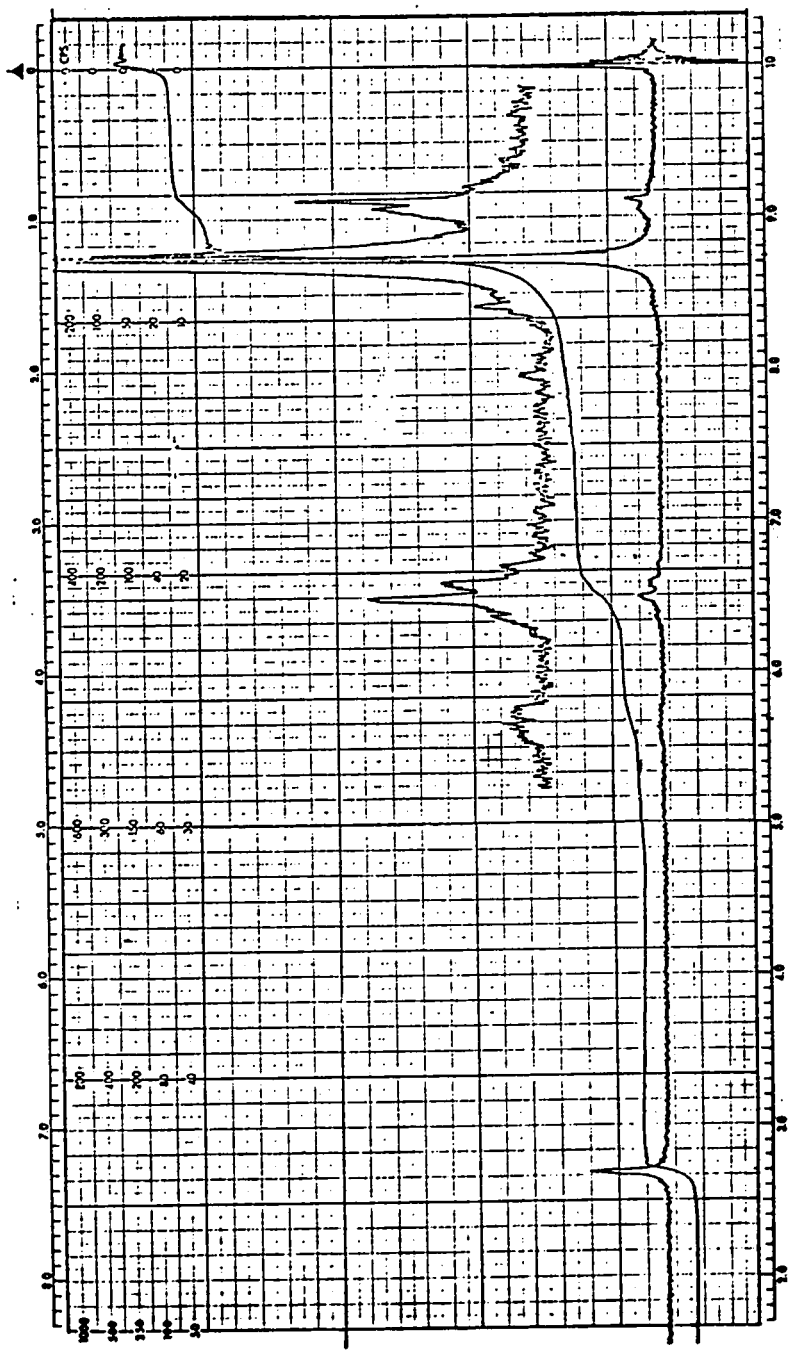

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 14. Nuclear magnetic resonance spectrum of 1,2-dioctadecyl-sn-glycero-3-diphenyl phosphoric acid. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


CPS
0
10
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
200
100
40
20
1000
400
200
100
40
20
800
400
200
100
50
30
1000
500
250
100
50
25

9.05 (2 x 3H); $-CH_2-C-$ 8.73 (64H): $-R-CH_2-O-CH-$, $R-CH_2-O-CH_2-$, $R-O-CH_2-CH$, $R-O-CH-CH_2$, 6.24 - 6.84 (7H); $-C-CH_2-OP$, 5.67 (2H); C_6H_5, 2.7 (2 x 5H) (Fig. 14). Anal. Calcd. for $C_{51}H_{89}O_6P$ (828); C, 73.91, H, 10.75; P, 3.74. Found C, 74.24; H, 10.81; P, 3.47.

2. 1,2-Dioctadecyl-sn-Glycero-3-Phosphoric Acid (Di-O-Octadecyl-L-α-Glycerophosphoric Acid)

2.4 g (0.003 moles) 1,2-dioctadecyl-sn-glycero-3-(diphenyl) phosphoric acid was dissolved in 120 ml of a mixture of chloroform and 99% ethanol (1:3 v/v). This solution was poured onto platinum catalyst (30), freshly prepared from 140 mg platinum oxide, and the mixture exhaustively hydrogenated at an initial pressure of 25 p.s.i. at room temperature (about 6 hours). The reaction mixture was filtered and the clear filtrate was evaporated to dryness under reduced pressure at 25 - 30°. The residue was dried in vacuo over P_2O_5 for three days at room temperature and washed with several portions of 30-80 ml acetone at 4°. The insoluble material was finally dried again in vacuo. The yield was 1.35 g (72% theory). This material could be reprecipitated from chloroform solution (50mg per ml) by dropwise addition of acetone; the analytical data were not altered by this treatment.

m.p. 68.5 - 69.5°, $[\alpha]_D^{25}$ = +1.28° (c = 5.07% w/v in chloroform). Values published subsequent to this work (37), m.p. 68 - 68.5°, $[2]_D^{20}$ +1.4°. Thin layer chromatography on Silica Gel G in either solvent A or solvent B showed a single spot on the origin. With chloroform;methanol:glacial acetic acid:water (80:13:8:0.3 v/v/v/v) as solvent and Silica gel H as absorbent, the material gave a single spot with R_F = 0.96. An aqueous dispersion (6mg ml^{-1}) had a pH = 3.2 (compare reference 154). Infrared absorption spectrum (NaCl pellet) (cm^{-1}): see Fig. 15. Nuclear magnetic

Figure 15. Infrared absorption spectra of 1,2-dioctadecyl-sn-glycero-3-phosphoric acid (A), 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine (B), and 1,2-dioctadecyl-sn-glycero-3-(methyl) phosphorylcholine (C). The spectra were taken as NaCl pellets using a Perkin-Elmer 621 spectrophotometer.

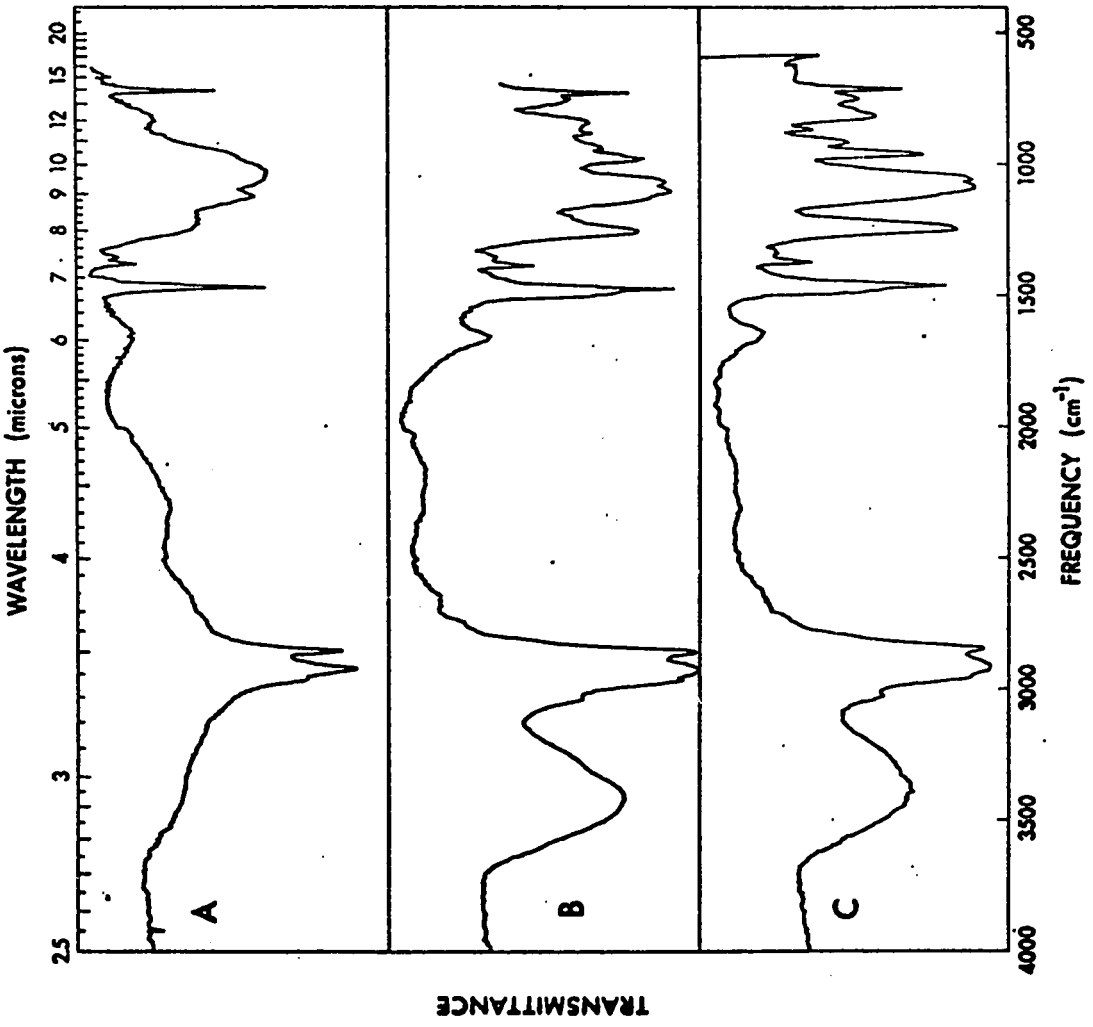
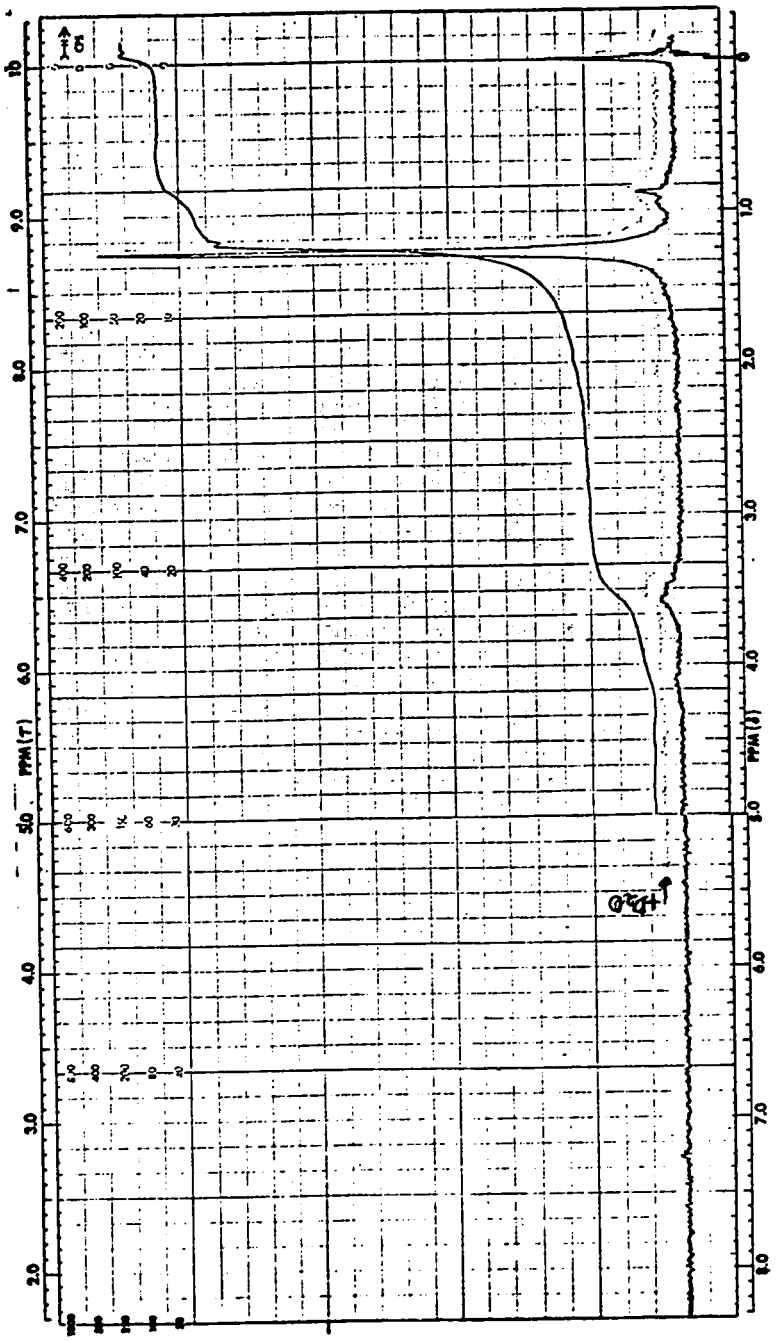

WAVELENGTH (microns)
2.5
3
4
5
6
7
8
9
10
12
15
20
FREQUENCY (cm⁻¹)
4000
3500
3000
2500
2000
1500
1000
500
A
B
C
TRANSMITTANCE

Figure 16. Nuclear magnetic resonance spectrum of 1,2-dioctadecyl-sn-glycero-3-phosphoric acid. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


PPM(T)
PPM(T)
H2O

resonance spectrum ($CDCl_3$) (τ): CH_3-C, 9.13, (2 x 3H); $-CH_2$-C-, 8.76, (2 x 32H); RCH_2-O-CH_2, R-CH_2-O-CH, R-O-CH_2-CH, R-O-CH-CH_2, 6.00 - 6.90, (7H); -C-CH_2-O-P, 5.75, (2H) (Fig. 16). Anal. Calcd. for $C_{39}H_{81}O_6P$ (677): C, 69.13; H, 11.96; P. 4.57. Found: C, 69.02; H, 11.89; P, 4.46.

3. 1,2-Ditetradecyl-sn-Glycero-3-Diphenylphosphoric Acid

The synthesis was based on that used to obtain 1,2-dioctadecyl-sn-glycero-3-diphenyl-phosphoric acid (35). 1,2-ditetradecyl-sn-glycerol (4.84 g 0.01 mole) in 62.5 ml anhydrous pyridine was mixed with a solution containing 2.96 g (0.011 mole) diphenylphosphorochloridate in 15 ml anhydrous pyridine. The reaction mixture was maintained under anhydrous conditions at 30°C for 20 hours. Residual diphenylphosphoro-chloridate was removed by addition of 1 ml of distilled water. Chopped ice (65 g) was added in small portions to precipitate the crude product. Then 400 ml ice cold distilled water was added and the mixture allowed to stand for 4 hours at 4°C.

The sticky precipitate was collected by filtration and washed with ice cold water in a cold room at 4°C. The solid material was dried in vacuo over P_2O_5 for at least 48 hours at room temperature. The dried material was extracted into 185 ml of warm petroleum ether (b.p. 37.6 - 50.9°C), 1 g of Celite was added and the insoluble material was removed by filtration. The filtrate was evaporated to remove the solvent. The residual oil was then dried in vacuo for several hours under P_2O_5.

The yield was 4.87 g (70% theory): $(n)_D^{26}$ 1.4795, $[\alpha]_D^{25}$ - 2.69 (C 7.5% w/v, in $CHCl_3$).

Thin-layer chromatography on Silica gel G with solvent A gave an R_F of 0.43.

Infrared absorption spectrum ($CHCl_3$) (cm^{-1}): C_6H_5-, 3020 (s, sharp), 1580 (s, sharp), 1470 (s, sharp); CH_3-, CH_2-, -CH-, 2910 (s), 2850 (s), 1455-, 1460 (s, doublet), 1370 (w); P = O, 1280 (s); P-O-C_6H_5, 1205 (s); C-O-C, 1105; P-O-C, 1185 (s), 1040 (m); other bands, 940 (s), 740 (s).

Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): CH_3-C- 9.12, 9.05 (2 x 3H): -CH_2-C-, 8.73 (48H); -R-CH_2-O-CH-, RCH_2-O-CH_2-, R-O-CH_2-CH, R-O-CH-CH_2, 6.0 - 6.9 (7H); -C-CH_2-O-P, 5.67 (2H); C_6H_5, 2.7 (2 x 5H). Anal. Calcd. for $C_{43}H_{73}O_6P$ (716.98): C, 72.03; H, 10.26; P, 4.32. Found: C, 71.86; H, 10.51; P, 4.12.

4. 1,2-Ditetradecyl-sn-Glycero-3-Phosphoric Acid

1,2-ditetradecyl-sn-glycero-3-diphenylphosphoric acid (4.07 g, 5.6 mmole) was dissolved in 120 ml of a mixture of 99% ethanol and chloroform (3:1 v/v). This solution was poured onto platinum catalyst (30) freshly prepared from 500 mg platinum oxide, and the mixture was exhaustively hydrogenated at an initial pressure of 25 p.s.i. at room temperature. After 8 hours, the reaction was complete, the reaction mixture was filtered and the clear filtrate was evaporated to dryness under reduced pressure. The dried residue was recrystallized from warm acetone (10 ml acetone/g lipid).

The yield was 3.0 g (90% theory):

m.p. 43 - 44°C, $[\alpha]_D^{25}$ + 1.6 (C, 5% w/v in $CHCl_3$).

Thin layer chromatography on Silica gel H with solvent C gave an R_F of 0.91.

Infrared absorption spectrum ($CHCl_3$) (cm^{-1}): CH_3-, CH_2-, -CH-, 2910 (s), 2850 (s), 1455 - 1460 (m, doublet); P = O, 1220; C-O-C, 1100; P-O-C, 1040 (m), other band, 940 (m).

Nuclear magnetic resonance ($CDCl_3$) (τ): CH_3-C, 9.13 (2 x 3H); -CH_2-C-, 8.76 (2 x 24H); RCH_2-O-CH_2, RCH_2-O-CH, R-O-CH_2-CH, R-O-CH-CH_2, 6.0 - 6.9 (7H); -C-CH_2-O-P, 5.75 (2H). Anal. Calcd. for $C_{31}H_{65}O_6P$ (564.80): C, 65.92; H, 11.60; P, 5.48. Found: C, 65.70; H, 11.72; P, 5.24.

5. 1,2-Dihexadecyl-sn-Glycero-3-Diphenylphosphoric Acid

The synthesis of this compound was the same as for 1,2-ditetradecyl-sn-glycero-3-diphenylphosphoric acid. The yield was 70% of theory. $[\eta]_D^{26}$ 1.4805, $[\alpha]_D^{25}$ - 2.61 (C, 8.5% w/v, in $CHCl_3$). Thin-layer chromatography on Silica gel G with solvent A gave an R_F of 0.4.

Infrared absorption spectrum ($CHCl_3$) (cm^{-1}) was identical with 1,2-ditetradecyl-sn-glycero-3-diphenylphosphoric acid.

Nuclear magnetic resonance ($CDCl_3$) (τ): CH_3-C, 9.12 (2 x 3H); -CH_2-C- 8.73 (56H); -R-CH_2-O-CH_2-, R-CH_2-O-CH_2-, R-O-CH_2-CH, R-O-CH-CH_2, 6.0 - 6.9 (7H); -C-CH_2-O-P, 5.67 (2H); C_6H_5, 2.7 (2 x 5H). Anal. Calcd. for $C_{47}H_{81}O_7P$ (772.67): C, 73.00; H, 10.57; P, 4.01. Found: C, 73.04; H, 10.48; P, 3.81.

6. 1,2-Dihexadecyl-sn-Glycero-3-Phosphoric Acid

The synthesis of this compound was similar to that of 1,2-ditetradecyl-sn-glycero-3-phosphoric acid except that the crude product was recrystallized from boiling acetone (15 ml acetone/g lipid). The yield was 90% theory.

m.p. 58 - 59°. $[\alpha]_D^{25}$ + 1.48 (C, 5.14% w/v, in $CHCl_3$).

Thin-layer chromatography on Silica gel H with solvent C gave an R_F of 0.91.

Infrared absorption spectrum ($CHCl_3$) (cm^{-1}) was identical with 1,2-ditetradecyl-sn-glycero-3-phosphoric acid.

Nuclear magnetic resonance ($CDCl_3$) (τ): CH_3-C, 9.13 (2 x 3H); $-CH_2-C-$, 8.76 (2 x 28H); RCH_2-O-CH_2, $R-CH_2-O-CH$, $R-O-CH_2-CH$, $R-O-CH-CH_2$, 6.0 - 6.9 (7H); $-C-CH_2-O-P$, 5.75 (2H). Anal. Calcd. for $C_{35}H_{73}O_6P$ (620.9): C, 67.70; H, 11.85; P, 4.98. Found: C, 67.52; H, 11.60; P, 5.11.

C. Synthesis of 1,2-Dialkyl-sn-Glycero-3-Phosphorylcholines (Scheme 8)

1. 1,2-Dioctadecyl-sn-Glycero-3-(Methyl) Phosphorylcholine (Di-O-Octadecyl-L-α-Glyceryl (Methyl) Phosphorylcholine)

0.99 g (0.0067 moles) methylphosphorodichloridate, 0.51 ml dry pyridine and 50 ml dry, ethanol-free chloroform were placed in a dry 300 ml, 2-necked flask, maintained at 10°. A solution of 3.98 g (0.0067 moles) 1,2-dioctadecyl-sn-glycerol in 50 ml dry, ethanol-free chloroform was added from a dropping funnel over a 45 minute period. The temperature was raised to 35° for 30 minutes, 8.3 ml dry pyridine was added and after 10-20 minutes 1.54 g choline iodide was added. The mixture was stirred with a magnetic stirrer at room temperature for

48 hours, filtered and the solvent removed from the filtrate by evaporation at 30 - 35°. Pyridine was removed as far as possible by drying *in vacuo* and the residue extracted with 6 x 50 ml anhydrous diethyl ether, 3 x 50 ml distilled water, 3 x 50 ml 99% ethanol and 3 x 50 ml acetone. The supernatants were removed after each washing by brief centrifugation at 10°. The dried residue was examined by thin layer chromatography on Silica gel G with solvent B and found to be quite heterogeneous. Consequently, the material was extracted several times with 50 ml portions of boiling acetone. After drying *in vacuo* 1.9 g of the residue was dissolved in 190 ml 95% ethanol-chloroform mixture (1:1 v/v). 2.4 g Ag_2CO_3 in 24 ml distilled water was then added and stirred for 70 minutes. The insoluble silver salts were removed by filtration and the filtrate evaporated to dryness under reduced pressure at 30 - 35°. The residue was dried overnight *in vacuo* over P_2O_5 at room temperature.

The iodide-free material in 30 ml chloroform was subjected to chromatography on a column (5 cm diameter) containing 100 g silicic acid (200 - 325 mesh) and 50 g celite. Elution was carried out with 500 ml chloroform, followed by 200 ml portions of chloroform-methanol mixtures containing gradually increasing proportions of methanol up to chloroform-methanol 7:3 v/v. This procedure removed two contaminants (R_F = 1 and R_F = 0) on thin layer chromatography on Silica gel G with solvent B. The silicic acid was then transferred from the chromatography tube into a large beaker and extracted with 5 x 400 ml chloroform-methanol (1:1 v/v). The supernatants were decanted and filtered and the combined filtrates evaporated to dryness at 35 - 40°.

The dry residue was redissolved in 15 - 20 ml chloroform and reprecipitated by slow addition of 100 ml anhydrous ether. The precipitate was collected and dried *in vacuo* for 12 hours at 60°. The yield was 0.65 g (11% theory based on 1,2-dioctadecyl-*sn*-glycerol).

m.p. 227 - 229° (sintering at 108°). $[\alpha]_D^{25}$ = +3.4° (c = 0.76% w/v in chloroform).

Thin-layer chromatography on Silica gel G with solvent B gave an R_F of 0.35. (egg-lecithin = 0.37, 1,2-dioctadecyl-*sn*-glycero-3-phosphorylcholine = 0.30).

Infrared absorption spectrum (NaCl pellet) (cm^{-1}): (See Fig. 15).

Nuclear magnetic resonance spectrum ($CDCl_3$) (τ): CH_3-C, 9.14 (2 x 3H); -CH_2-C, 8.76 (64H); -$N(CH_3)_3$, 6.63 (3 x 3H); -$N(CH_3)_3$ after shaking with D_2O, 6.81 (3 x 3H); RCH_2-O-CH_2, RCH_2-O-CH, R-O-CH_2-CH, R-O-CH-CH_2, 6.30 - 6.70 (7H); CH_3-O-P, 6.16, 6.34 (3H); -CH_2-N-, 6.16 (2H); -CH_2-O-P, 5.75 (2H). Anal. Calcd. for $C_{45}H_{96}O_7NP$ (794): C, 68.05, H; 12.19; N, 1.76; P, 3.90. Found: C, 67.80; H, 11.98; N, 1.76; P, 3.98, 3.83.

2. 1,2-Dimethyl-*sn*-Glycero-3-Phosphorylcholine (Di-O-Methyl-L-α-Glycerylphosphorylcholine

5.70 ml (0.038 moles) monophenylphosphorodichloridate, 3.08 ml (0.038 moles) pyridine and 80 ml dry, ethanol-free chloroform were placed in a dry 300 ml, 2-necked flask maintained at 10°. A solution containing 4.57 g (0.038 moles) 1,2-dimethyl-*sn*-glycerol in 200 ml of dry ethanol-free chloroform was added from a dropping funnel over a 2 hour

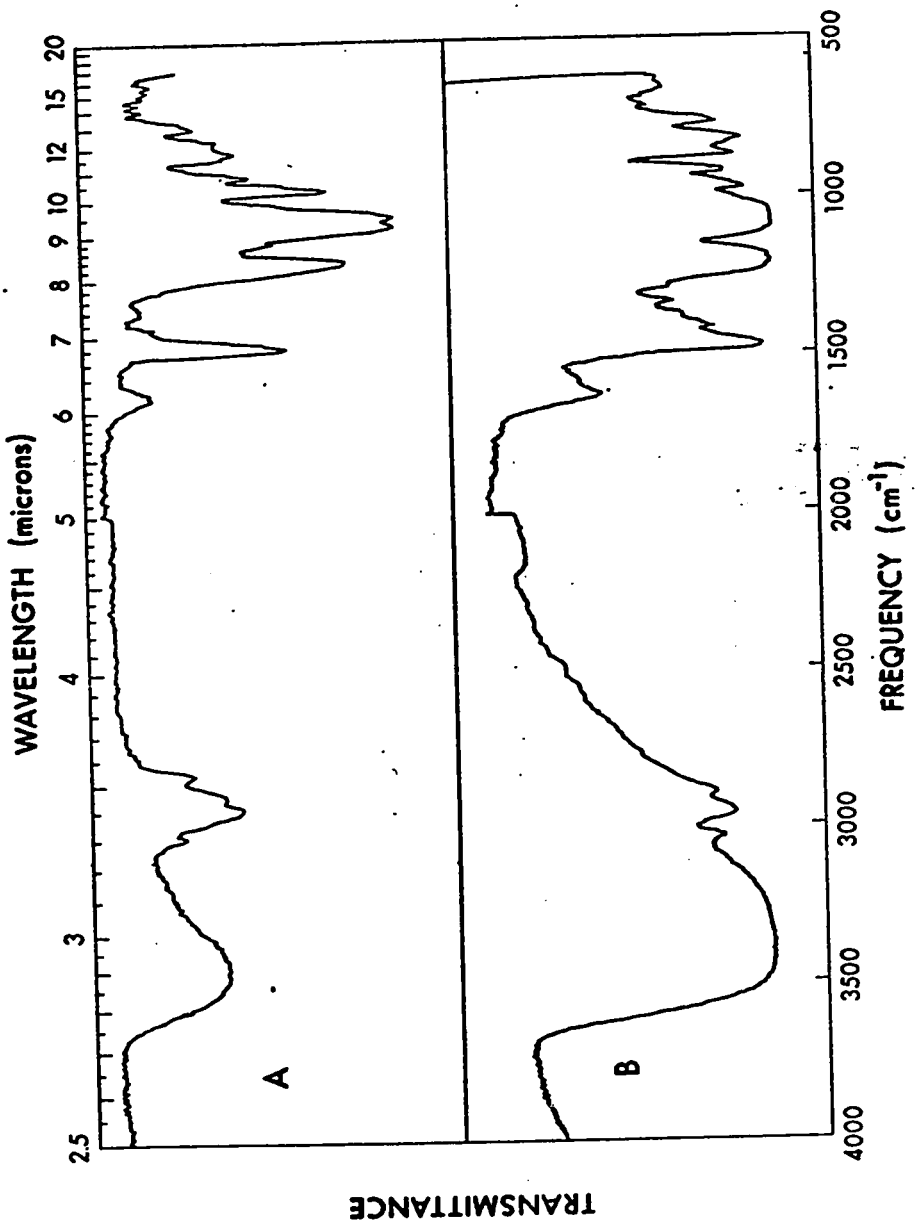
period. The temperature was raised to 35° for 30 minutes, 50 ml dry pyridine was added and after 10 - 20 minutes, 8.78 g choline iodide (0.038 moles) was added. The mixture was stirred at room temperature for 58 hours, filtered and the slightly yellow, clear filtrate evaporated to dryness at 30 - 35°. The residue,still containing some pyridine, was then dissolved in one liter 95% ethanol and 21 g Ag_2CO_3 in 210 ml distilled water was added. The mixture was stirred with a magnetic stirrer at room temperature for two hours. Insoluble material was removed by filtration and the filtrate taken to dryness by evaporation for several hours at 30 - 35°. Any black precipitate observed during evaporation was removed by filtration. The residue, a brown oil, was dried *in vacuo* over P_2O_5 at room temperature, dissolved in 120 ml 99% ethanol and subjected to 25 p.s.i. hydrogen over 0.81 g freshly prepared platinum catalyst (30). Very little uptake of hydrogen was observed under these conditions. At this point, 30 ml chloroform was added and the mixture again hydrogenated at an initial pressure of 25 p.s.i. at room temperature. 0.036 moles hydrogen were absorbed within 5 - 6 hours. After hydrogenolysis the catalyst was removed by filtration and the clear colorless filtrate evaporated to dryness. The residue was washed with 7 x 350 ml anhydrous diethyl ether and dissolved in 200 ml methanol-chloroform (1:9 v/v). Thin layer chromatography of this material on Silica gel H with solvent B revealed six components with R_F values: 0.95, 0.57, 0.43, 0.24, 0.10 and 0. The component with R_F = 0.24 appeared as a white spot on a brown background after spraying with iodine.

150 g silic acid (100 mesh) and 75 g celite were mixed, suspended in chloroform and poured into a chromatography tube 5 cm in diameter. The reaction mixture was applied on the column and elution carried out with several liters of methanol-chloroform (1:9 v/v). This solvent removed components with R_F = 0.95, 0.57, 0.24, and 0. Elution was continued with 1 - 2 liters methanol-chloroform (1:1 v/v), the eluates combined and the solvent removed by evaporation. To convert remaining traces of 1,2-dimethyl-sn-glycero-3-(phenyl) phosphorylcholine to 1,2-dimethyl-sn-glycero-3-phosphorylcholine the dry residue was dissolved in 120 ml 99% ethanol-chloroform (3:1 v/v) and again subjected to 25 p.s.i. hydrogen for 5 - 6 hours in the presence of 300 mg freshly prepared platinum catalyst (30). The catalyst was removed by filtration, washed with 10 - 20 ml 99% ethanol and the combined filtrates evaporated to dryness. The dry residue was re-dissolved in 50 ml 99% ethanol and any insoluble material removed by filtration. Thin layer chromatography on Silica gel G with solvent B revealed a single component with R_F = 0.07. After removal of the solvent,2 g 1,2-dimethyl-sn-glycero-3-phosphorylcholine was obtained (18% theory). This compound is a hygroscopic, viscous oil, soluble in water and ethanol but insoluble in chloroform and ether. It was therefore converted to the cadmium chloride complex.

3. 1,2-Dimethyl-sn-Glycero-3-Phosphorylcholine Cadmium Chloride Complex. (Di-O-Methyl-L-α-Glycerophosphorylcholine Cadmium Chloride Complex).

72 mg of 1,2-dimethyl-sn-phosphorylcholine (0.24 mmoles) was dissolved in 5.4 ml 95% ethanol. 151.2 mg $CdCl_2 \cdot 2.5\ H_2O$ was dissolved in 0.081 ml distilled water, diluted with 1.81 ml 99% ethanol (153)

Figure 17. Infrared absorption spectra of the (1,3-dimethyl-sn-glycero-3-phosphorylcholine)$_2$ $(CdCl_2)_3$ complex (A) and the (sn-glycero-3-phosphorylcholine)$_2(CdCl_2)_3$ complex (B). The spectra were taken as NaCl pellets using a Perkin-Elmer 621 spectrophotometer.


WAVELENGTH (microns)
2.5
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FREQUENCY (cm⁻¹)
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2500
2000
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1000
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A
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TRANSMITTANCE

and added slowly with stirring (magnetic stirrer) to the solution of 1,2-dimethyl-sn-glycero-3-phosphorylcholine. A precipitate formed immediately at room temperature. The mixture was kept at 0 - 4° for one hour and the precipitate was collected by centrifugation and washed at room temperature with three portions of 15 - 20 ml 99% ethanol and with three portions 15 - 20 ml anhydrous diethyl ether. The amorphous solid was dried in vacuo at 65° overnight. The yield of (1,2-dimethyl-sn-glycero-3-phosphorylcholine)$_2$ $(CdCl_2)_3$ was 110 mg (80% theory).

$[\alpha]_D^{25}$ = +3.9° (c = 1.66% w/v in 50% ethanol). Infrared absorption spectrum: (NaCl pellet) (cm^{-1}): (Fig. 17). Anal. Calcd. for $C_{20}H_{52}O_{14}N_2P_2Cd_3Cl_6$ (1130): C, 21.26; H, 4.63; N, 2.48; P, 5.48. Found: C, 21.68; H, 4.24; N, 2.86; P, 5.27.

4. 1,2-Dihexadecyl-sn-Glycero-3-Phosphorylcholine

This compound was prepared from 1,2-dihexadecyl-sn-glycerol essentially as described by Stanacev, Baer and Kates (30) for the synthesis of 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine. A typical preparation was carried out as follows:

(a) 1,2-dihexadecyl-sn-glycero-3-phosphoryl(phenyl)choline iodide

Monophenylphosphorodichloridate (2 ml, 15 mmole), 1.13 ml anhydrous pyridine, and 30 ml of dry, ethanol-free chloroform were placed in a dry 300 ml, two-necked flask, maintained at 10°C. A solution of 7.2 g (15 mmole) 1,2-dihexadecyl-sn-glycerol in 75 ml of dry chloroform was added from a dropping funnel over 80 minutes. The temperature was raised to 35°C within 15 minutes and after 60 minutes 16.7 ml of anhydrous pyridine were added. This was followed after 20

minutes by the addition of 3.08 g (15 mmole) of choline iodide. The mixture was stirred with a magnetic stirrer at room temperature for 60 h, filtered and the solvent removed from the filtrate by evaporation at 30 - 35°C. Pyridine was removed as far as possible by drying *in vacuo* and the dry residue was washed with 8 x 75 ml anhydrous ether, each time separating the mixture with brief centrifugation at 0°C. The ether-insoluble material was dried *in vacuo* and then washed successively with 3 x 35 ml of water, 3 x 35 ml 99% ethanol and 3 x 35 ml acetone, again separating the mixture by centrifugation and discarding the supernatant. The solid material (after acetone-washing) was dissolved in 70 ml of boiling acetone, the solution was filtered while hot and allowed to crystallize at 4°C overnight. The crystalline material weighed 3.85 g (30% of theory).

$[\alpha]_D^{25}$ - 3.8 (c 10% w/v/ in chloroform) m.p. 143 - 144°C.

Thin-layer chromatography on Silica gel H with solvent B gave an R_F of 0.54.

Infrared absorption spectrum: (Fig. 18).

Nuclear magnetic resonance spectrum: (Fig. 19).

Phosphorus analysis: Found P = 3.46% (calculated value = 3.43%).

(b) Removal of Iodide Ion and Phenyl Group

3.7 g (4.0 mmole) of 1,2-dihexadecyl-*sn*-glycero-3-phosphoryl (phenyl) choline iodide was dissolved in 360 ml of a mixture 95% ethanol-chloroform (3:1 v/v) and 5.0 g Ag_2CO_3 in 45 ml distilled water were added. The mixture was stirred at room temperature for 2 hours and filtered and the filtrate was evaporated to dryness at 30 - 35°C. The residue was dissolved in 120 ml of a mixture 99% ethanol and chloroform (3:1 v/v) and subjected to 25 p.s.i. hydrogen

Figure 18. Infrared absorption spectrum of 1,2-dihexadecyl-sn-glycero-3-phosphoryl(phenyl) choline iodide. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophotometer.

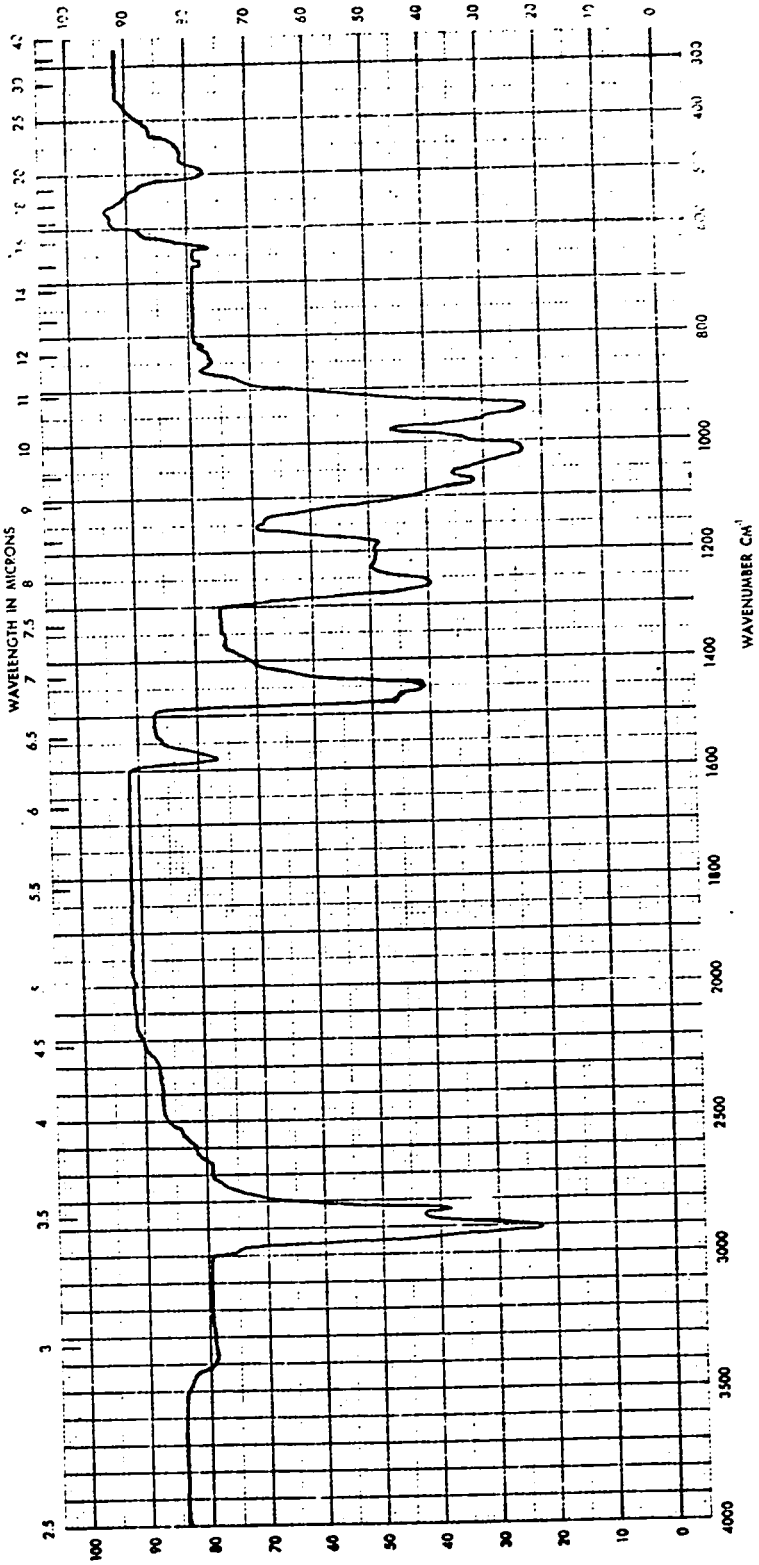
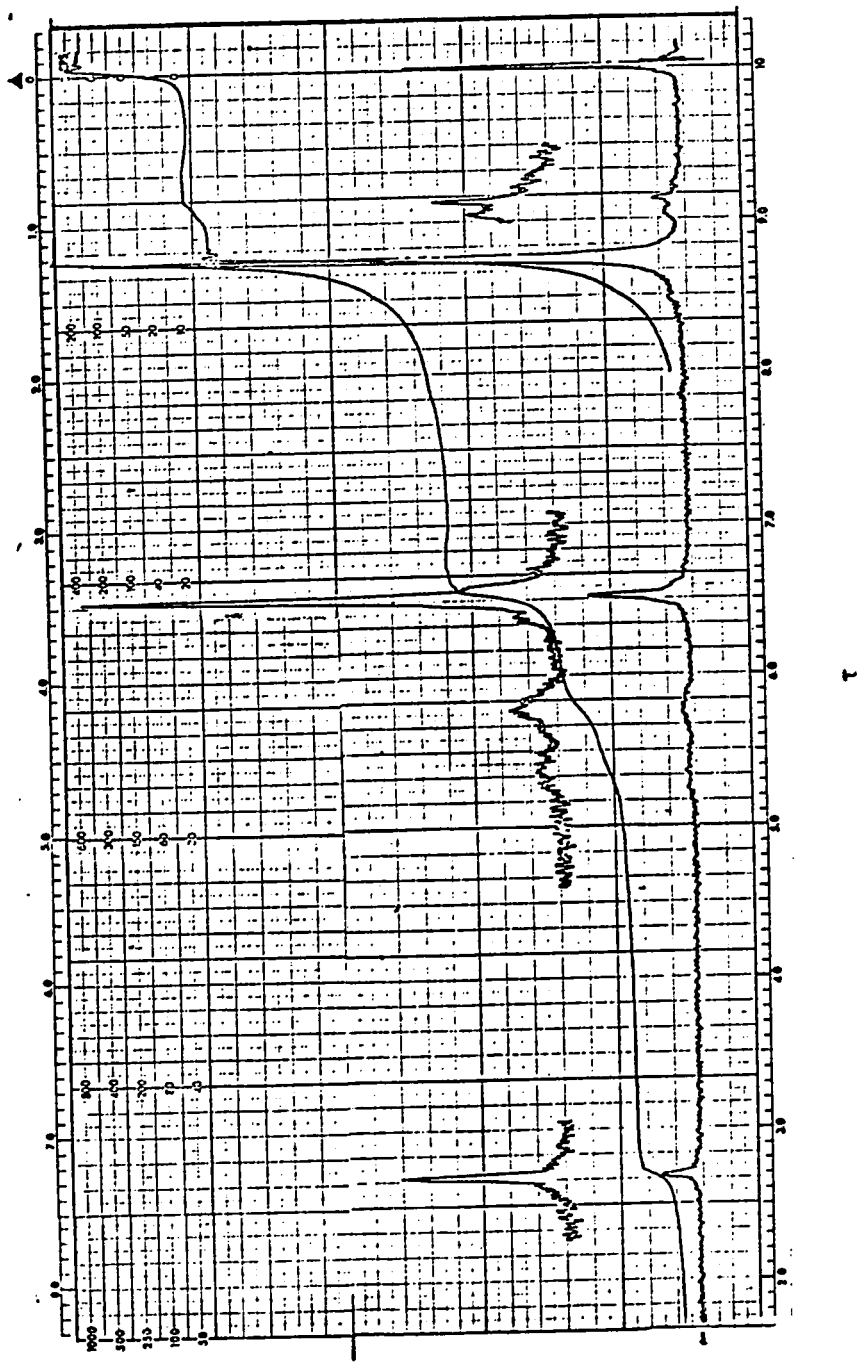

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 19. Nuclear magnetic resonance spectrum of 1,2-dihexadecyl-sn-glycero-3-phosphoryl (phenyl) choline iodide. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.



over 0.5 g freshly prepared platinum catalyst (30). The reaction was complete in 6 hours. After hydrogenolysis, the catalyst was removed by filtration and the clear colorless filtrate was evaporated to dryness. The residue was dissolved in 10 ml of chloroform and was further purified with silicic acid column chromatography (5 cm diamter, 150 g silicic acid, 100 mesh and 75 g celite). The column was first washed with 1.2 liters of 5% methanol in chloroform to remove the fast moving components and then washed with 1.5 liters of 25% methanol in chloroform to elute the desired compound. The desired fractions were combined and the solvent was removed with reduced pressure. The residue was dried in vacuo over P_2O_5. The yield of 1,2-dihexadecyl-sn-glycero-3-phosphorylcholine was 1.5 g (21% of theory).

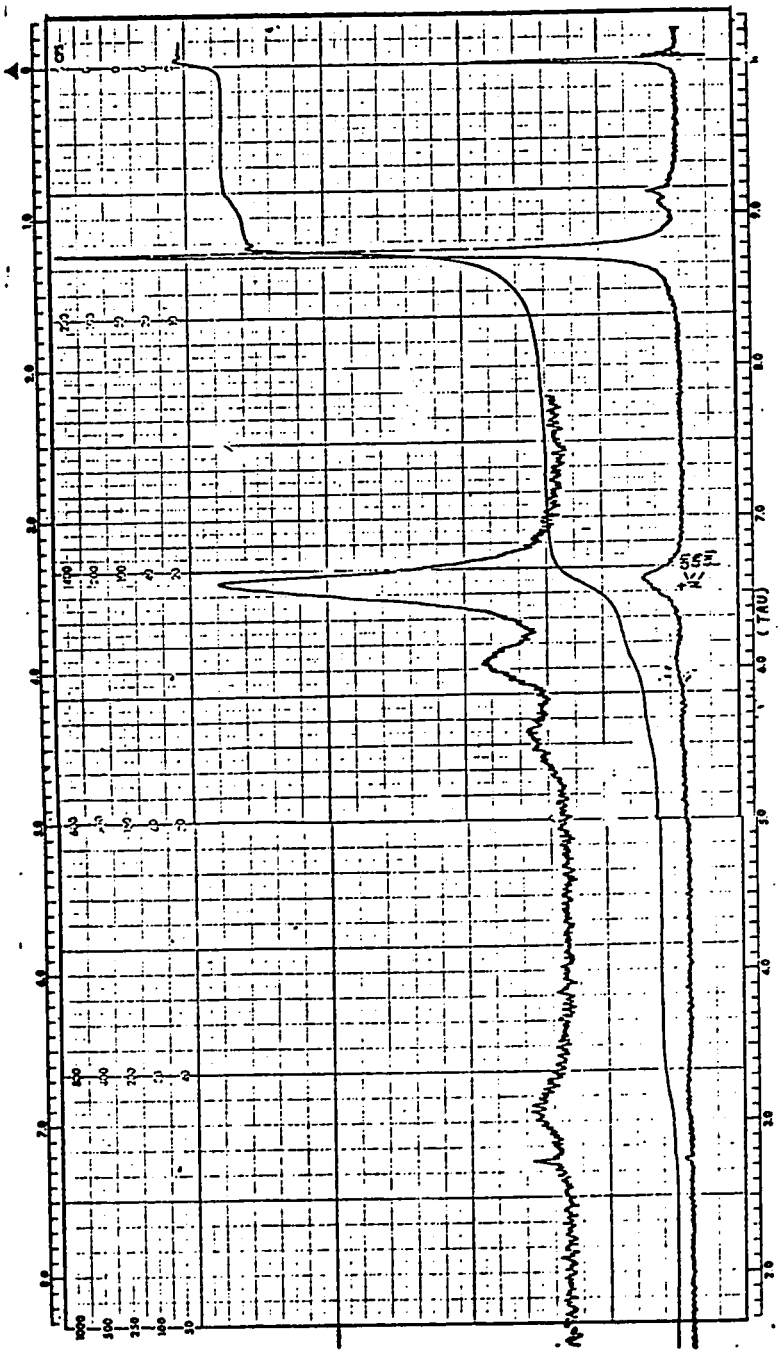
m.p.: 211 - 212°C. $[\alpha]_D^{25}$ + 1.40 (C, 5% w/v, in $CHCl_3$:CH_3OH 1:1 v/v).

Thin-layer chromatography on Silica gel G with solvent B gave an R_F of 0.39.

The infrared absorption spectrum ($CHCl_3$) (cm^{-1}) was identical with that of 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine (Fig. 15).

Nuclear magnetic resonance ($CDCl_3$) (τ): CH_3 -, 9.12 (2 x 3H); $-CH_2-C$, 8.76 (2 x 28H); $N(CH_3)_3$, 6.63 (3 x 3H); $-N(CH_3)_3$ after shaking with D_2O, 6.81 (3 x 3H); RCH_2-O-CH_2, RCH_2-O-CH, $R-O-CH_2-CH$, $R-O-CH-CH_2$, 6.30 - 6.70 (7H); $-CH_2-N-$, 6.16 (2H); $-CH_2-O-P$, 5.75 (4H) (Fig. 20). Anal. Calcd. for $C_{40}H_{86}O_7NP$ (724.07): C, 66.34; H, 11.97; N, 1.93; P, 4.27. Found: C, 65.88; H, 11.76; N, 1.88; P, 4.40.

Figure 20. Nuclear magnetic resonance spectrum of 1,2-dihexadecyl-sn-glycero-3-phosphorylcholine. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.



5. 1,2-Ditetradecyl-sn-Glycero-3-Phosphorylcholine

This compound was synthesized from 1,2-ditetradecyl-sn-glycerol essentially as described for 1,2-dihexadecyl-sn-glycero-3-phosphorylcholine.

m.p.: 212 - 213°C $[\alpha]_D^{25}$ + 1.55 (C, 5.07% w/v, in $CHCl_3$: CH_3OH 1:1 v/v).

Thin-layer chromatography with solvent B gave an R_F of 0.39.

The infrared absorption spectrum ($CHCl_3$) (cm^{-1}) was identical with 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine (Fig. 15).

Nuclear magnetic resonance ($CDCl_3$) (τ): CH_3-, 9.12 (2 x 3H): $-CH_2-C$, 8.76 (2 x 24H); $N(CH_3)_3$, 6.63 (3 x 3H); $N(CH_3)_3$ after shaking with D_2O, 6.81 (3 x 3H); RCH_2-O-CH_2; RCH_2-O-CH, $R-O-CH_2-CH$, $R-O-CH-CH_2$, 6.30 - 6.70 (7H); $-CH_2-N-$, 6.16 (2H); $-CH_2-O-P$, 5.75 (4H). Anal. Calcd. for $C_{36}H_{78}O_7NP$ (667.96): C, 64.73; H, 11.77; N, 2.09; P, 4.64. Found: C, 63.85; H, 11.48; N, 2.03; P, 4.64.

6. Disodium Salt of 1,2-Ditetradecyl-sn-Glycero-3-Phosphoric Acid

This was prepared according to Reference 138.

The physical properties of synthetic dialkyl ether lipids were shown in Table I.

D. Discussion

Some differences in technique in preparing the methyl diethers as compared with the long chain diethers may be commented upon. For example, 1,2-dimethyl-3-benzyl-sn-glycerol is a liquid at room temperature and reacts very much more rapidly with hydrogen in the presence of palladium on charcoal than does 1,2-dioctadecyl-3-benzyl-sn-glycerol. Again, the intermediate, 1,2-dimethyl-sn-glycero-3-(phenyl) phosphorylcholine iodide possesses quite different solubility

characteristics from the dioctadecyl analog and could therefore not be isolated in pure form by the method of reference (30). 1,2-dimethyl-sn-glycero-3-phosphorylcholine is a very hygroscopic liquid and is therefore best characterized as the cadmium chloride adduct. The latter compound has the same stoichiometry as the analogous sn-glycero-3-phosphorylcholine (L-α-glycerophosphorylcholine) cadmium chloride complex and like it is an amorphous or microcrystalline solid.

With the exception of 1,2-dioctadecyl-sn-glycero-3-phosphate (Fig. 15A), all the infrared spectra shown in Figs. 15 and 17 show a strong, broad band from 3,300 - 3,500 cm^{-1}. Since choline-containing lipids as well as quaternary ammonium paraffin-chain salts are much more hygroscopic than the phosphatidic acid analog it seems likely that this band is due to the presence of water associated with quaternary ammonium groups. This conclusion is supported by the n.m.r. data discussed below. The infrared spectra shown in Figs. 15B and 15C, respectively, are closely similar to each other. The distinguishing features are the presence of weak absorption bands at 2,650 cm^{-1} and 2,680 cm^{-1} due to the phosphorus oxyacid group in the diester and the location of the C-H stretching vibrations at 2,860 cm^{-1} and 2,845 cm^{-1} in the diester and triester respectively. In Fig. 17 the spectra of (1,2-dimethyl-sn-glycero-3-phosphorylcholine)$_2$ $(CdCl_2)_3$ and (sn-glycero-3-phosphorylcholine)$_2$ $(CdCl_2)_3$ are compared. The dimethyl compound shows two bands at 1090 cm^{-1} (C-O-C) and 1055 cm^{-1} (P-O-C) whereas only one broad band (P-O-C) is evident in the spectrum of the glycerylphosphorylcholine adduct. In both compounds the absorption for P = O is at 1200 cm^{-1} as compared with 1250 cm^{-1} for P = O in the spectra

shown in Figs. 15A and 15B, suggesting that the P = O group is implicated in the formation of the cadmium chloride complex.

Nuclear magnetic resonance spectra were used for partial characterization of several intermediates and products. While the spectra have been extremely useful in distinguishing reactants and products in various steps they have not always been completely definitive. A major reason for this is the overlapping of resonances from the groups $R-CH_2-O-CH_2$, $R-CH_2-O-CH$, $R-O-CH_2-CH$, and $R-O-CH-CH_2$. The resulting signals are more complex than those arising from the corresponding diacyl compounds (155). Other functional group assignments are in excellent agreement with those given by Chapman and Morrison (155) for diacyl compounds. It was also observed that the signal for $-N(CH_3)_3^+$ reproducibly shifted upfield by about 0.2 p.p.m. on shaking solutions of either 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine or 1,2-dioctadecyl-sn-glycero-3-(methyl)phosphoryl-choline in $CDCl_3$ with D_2O. This observation may be indicative of the shielding effect of a hydration shell around the quaternary ion under these conditions.

E. Summary

The dialkyl ether phospholipids synthesized were: 1,2-dioctadecyl-sn-glycero-3-phosphoric acid; 1,2-dihexadecyl-sn-glycero-3-phosphoric acid; 1,2-ditetradecyl-sn-glycero-3-phosphoric acid; 1,2-dioctadecyl-sn-glycero-3-(methyl)-phosphorylcholine; 1,2-dimethyl-sn-glycero-3-phosphorylcholine; 1,2-dihexadecyl-sn-glycero-3-phosphorylcholine and 1,2-ditetradecyl-sn-glycero-3-phosphorylcholine.

TABLE I

PHYSICAL PROPERTIES OF DIALKYL ETHER LIPIDS

Substance	M.P.	$[\alpha]_D^{25}$ [a]	$[M]_D$ [a]
1,2-Dimethyl-sn-glycerol		-5.27 [b]	- 6.32
1,2-Ditetradecyl-sn-glycerol	42.5 - 43.5	-8.0	-39.0
1,2-Dihexadecyl-sn-glycerol	48.5 - 49.5	-7.5	-41.0
1,2-Dioctadecyl-sn-glycerol	56.0 - 57.5	-6.9	-41.0
1-(Bis-9,10-cis-dibromo) octadecyl-2-octadecyl-sn-glycerol	-	-4.9	-37.0
1,2-Ditetradecyl-sn-glycero-3-phosphoric acid	43.0 - 44.0	+1.6	+ 9.0
1,2-Dihexadecyl-sn-glycero-3-phosphoric acid	58.0 - 59.0	+1.48	+ 9.1
1,2-Dioctadecyl-sn-glycero-3-phosphoric acid	68.5 - 69.5	+1.28	+ 8.6
(1,2-Dimethyl-sn-glycero-3-phosphorylcholine)$_2$($CdCl_2$)$_3$	-	+3.9 [c]	+44.0
1,2-Ditetradecyl-sn-glycero-3-phosphorylcholine	212.0 - 213.0	+1.55 [d]	+10.4
1,2-Dihexadecyl-sn-glycero-3-phosphorylcholine	211.0 - 212.0	+1.40 [d]	+10.1
1,2-Dioctadecyl-sn-glycero-3-phosphorylcholine	201.0 - 202.0	+1.30 [d]	+10.1
1,2-Dioctadecyl-sn-glycero-3-phosphoryl(methyl)choline	227.0 - 229.0	+3.4 [d]	+27.0

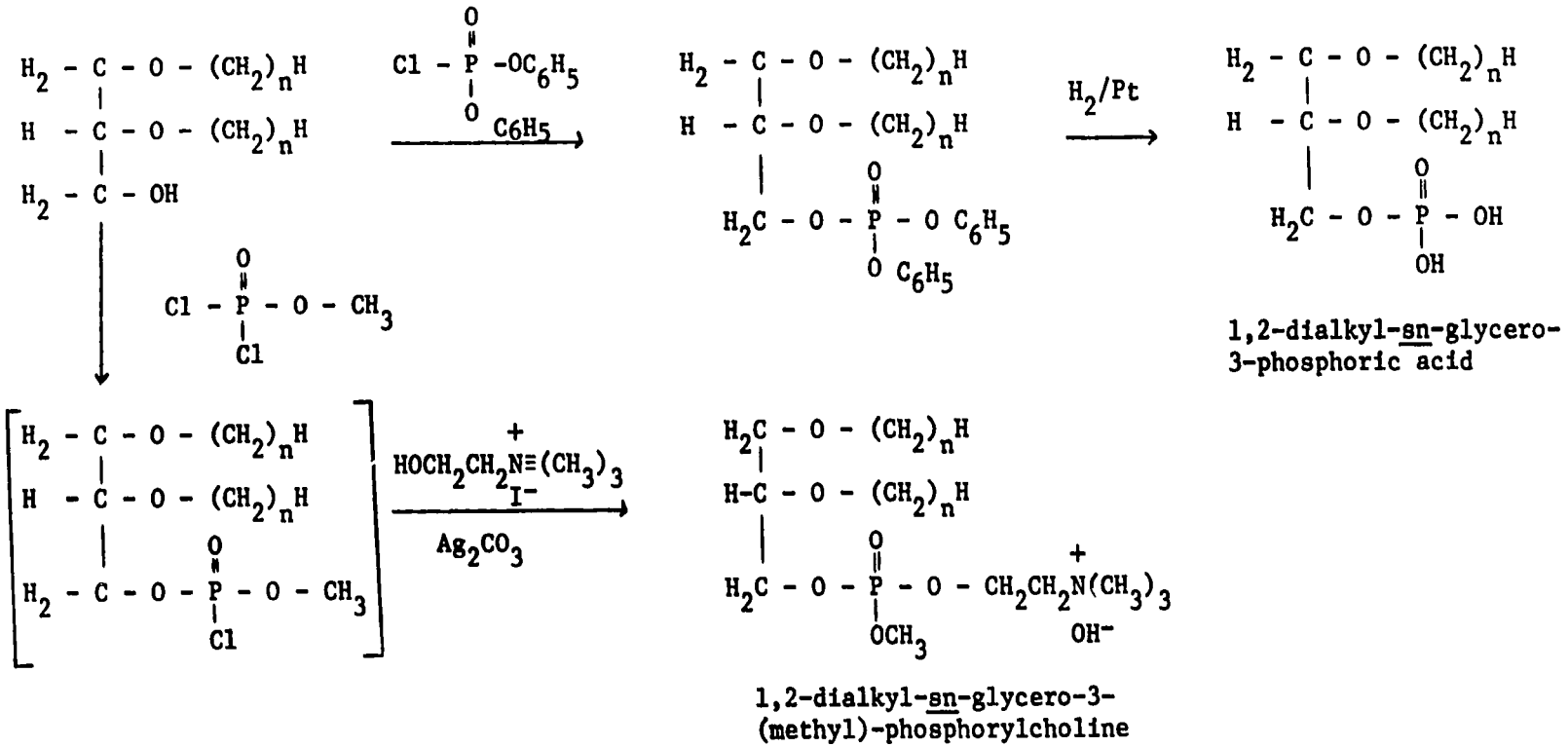
[a] in chloroform

[b] pure liquid

[c] solvent 50% ethanol in water

[d] solvent chloroform:methanol 1:1 (v/v).

Scheme 7. Synthesis of 1,2-dialkyl-*sn*-glycero-3-phosphoric acid and 1,2-dialkyl-*sn*-glycero-3-(methyl) phosphorylcholine.

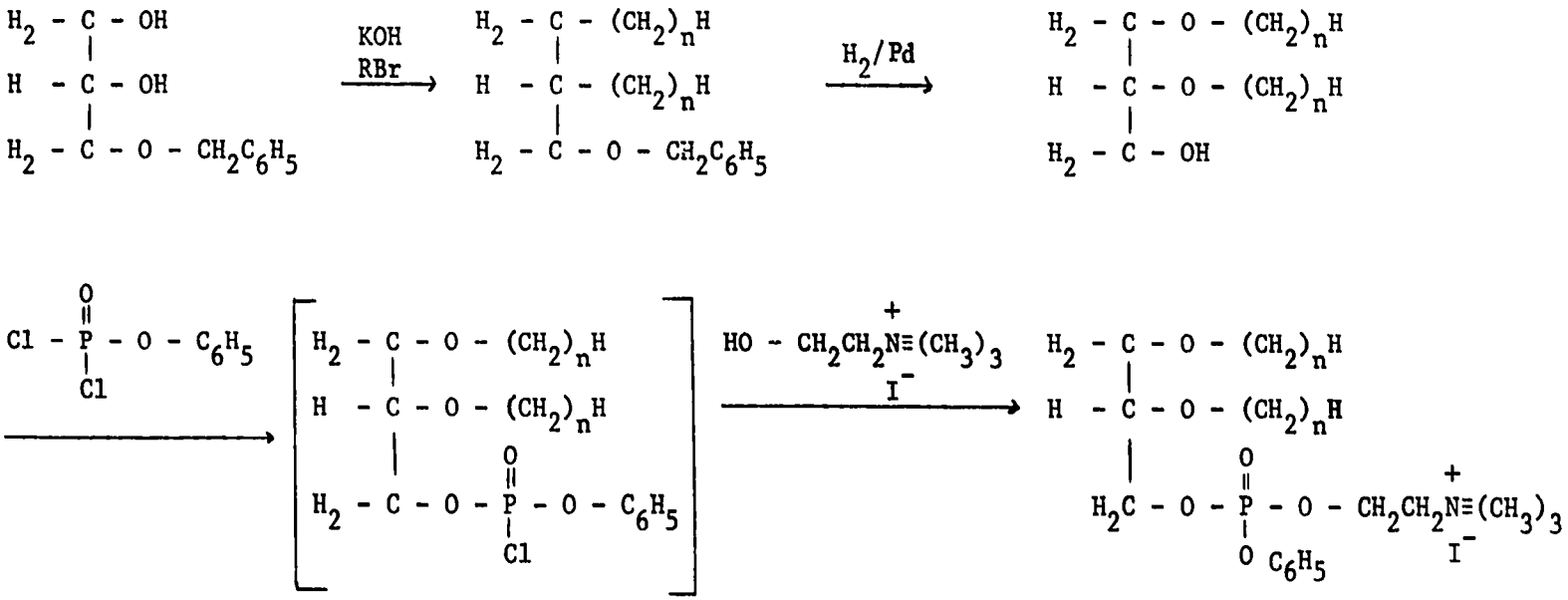


where n = 18 (octadecyl)

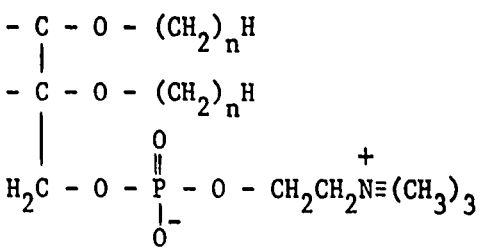
n = 16 (hexadecyl)

n = 14 (tetradecyl)

Scheme 8. Synthesis of 1,2-dialkyl-sn-glycero-3-phosphorylcholine.



1. Ag_2CO_3
2. H_2/Pt



1,2-dialkyl-sn-glycero-3-phosphorylcholine

1,2-dialkyl-sn-glycero-3-phosphoryl(phenyl)choline iodide

where n = 18 (octadecyl)

n = 16 (hexadecyl)

n = 14 (tetradecyl)

n = 1 (methyl)

CHAPTER IV

ENZYMIC HYDROLYSIS OF DIALKYL ETHER PHOSPHOLIPIDS BY CABBAGE LEAF PHOSPHOLIPASE D

It had been reported that the activity of soluble phospholipase D from cabbage leaf was stimulated by diethyl ether (106, 112) and anionic amphipathic substances (106). In the following experiments, the effect of diethyl ether, anionic detergent (sodium dodecyl sulfate), nonionic detergent (BRIJ 58, polyoxyethylene cetyl ether), cationic detergent (CTABr, cetyltrimethylammonium bromide) and phosphatidic acids on the phospholipase D hydrolysis of dialkyl-sn-glycero-3-phosphorylcholines was investigated.

A. Hydrolysis of Dialkyl Ether Phospholipids using Organic Solvents as Activators

It was found that all of the dialkyl ether phospholipids were relatively insoluble in $(C_2H_5)_2O$ but dissolved readily in $CHCl_3$. Since $(C_2H_5)_2O$:$CHCl_3$ 10:1 v/v was reported to be a better activator of egg lecithin hydrolysis than $(C_2H_5)_2O$ alone (112), the mixed solvent was used in most experiments.

In the absence of organic solvent, none of the dialkyl ether phospholipids (14C EPC, 16C EPC, 18C EPC) was hydrolyzed by phospholipase D. In the presence of $(C_2H_5)_2O$-$CHCl_3$ (10:1 v/v) and at low enzyme concentrations, again no hydrolysis was observed (Fig. 21a). However, at high enzyme concentrations, the dialkyl ether phospholipids were slowly hydrolyzed (Fig. 21b).

Figure 21a. Hydrolysis of hand-shaken dispersions of phospholipids by phospholipase D. Standard reaction mixture A. Type II enzyme (1 mg/ml) and $(C_2H_5)_2O$:$CHCl_3$ 10:1 v/v were used. ●, egg lecithin; ▲, 18C EPC; Δ, hydrogenated egg lecithin; ▫, 14C EPC. (13 μmoles)

Figure 21b. Hydrolysis of 14C EPC and 18C EPC by phospholipase D. Standard reaction mixture A. Type I enzyme , lot no. 20C - 2090 was used. Incubated at 26°C for 1 hour. ●, 14C EPC; ▲, 18C EPC; ▫, 16 μmoles egg lecithin and Type I enzyme (1 mg/ml) were used. Incubated at 26°C for 10 minutes. ■, 16 μmoles egg lecithin and Type I enzyme (7 mg/ml) were used. Incubated at 26°C for 10 minutes.

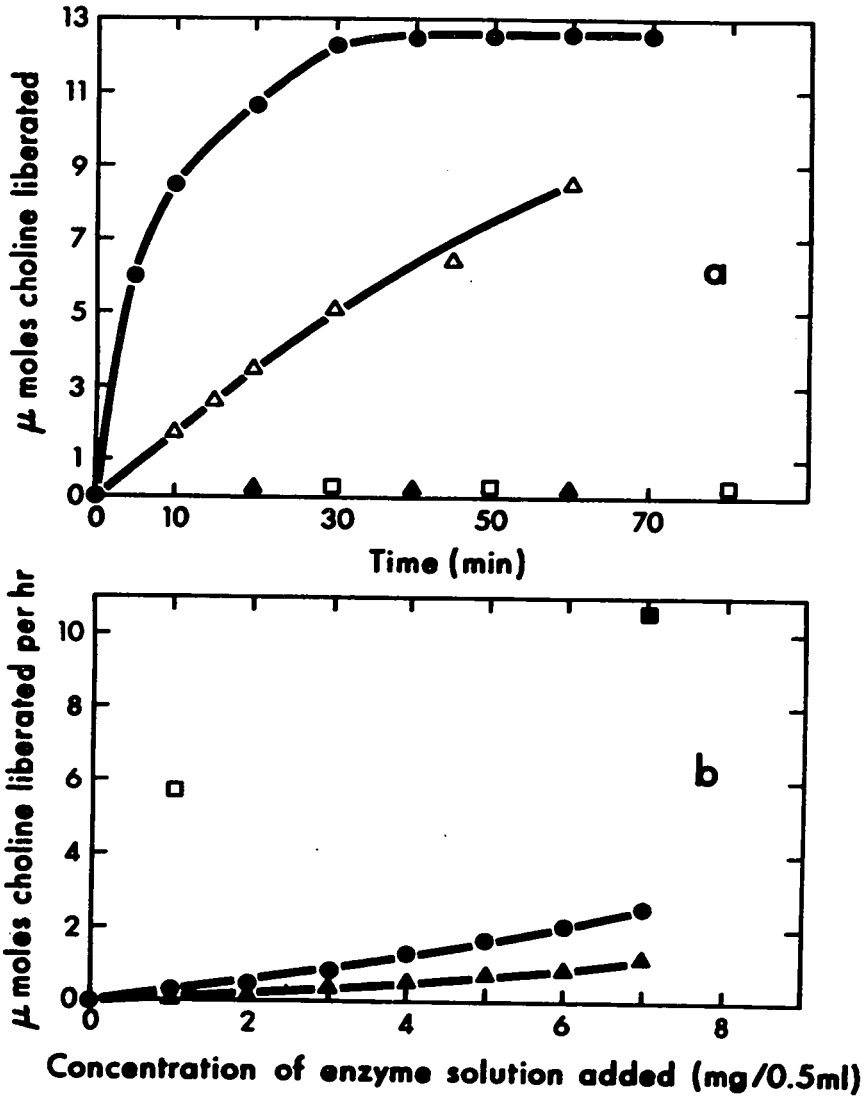

μ moles choline liberated
13
11
9
7
5
3
1
0
0
10
30
50
70
Time (min)
a
μ moles choline liberated per hr
10
8
6
4
2
0
0
2
4
6
8
b
Concentration of enzyme solution added (mg/0.5ml)

Table II shows the relative rates of hydrolysis of egg lecithin, hydrogenated egg lecithin and the dialkyl ether phospholipids.

In view of the rather low rates of hydrolysis of the dialkyl ether phospholipids in comparison with their diacyl homologs (112), it was considered that differences in their solubilities in the organic solvent might be important. Therefore, the partition of the various phospholipids between the organic solvent and the aqueous phase was studied.

Standard reaction mixture A (half-quantities) was used with Type I enzyme (0.5 mg/0.5 ml) and with the solvent $(C_2H_5)_2O$:$CHCl_3$ 10:1 v/v. The stoppered tubes were rapidly mixed for 10 seconds, then centrifuged immediately at top speed in an International Clinical Centrifuge for about 30 seconds. The ethereal layer and aqueous layer were separated and total P_i was determined in each phase. Each result was the average of duplicate experiments expressed as mole % present in the organic phase. The values thus obtained were egg-lecithin, 98%; hydrolecithin, 89%; 14C EPC, 86%; 16C EPC, 85% and 18C EPC, 76%. Therefore, it seems that differences in solubility of different phospholipids in $(C_2H_5)_2O$:$CHCl_3$ (10:1 v/v) could only partially account for their different rates of hydrolysis.

B. Locus of Enzymic Hydrolysis

Kates (128) demonstrated that the hydrolysis of egg lecithin catalyzed by phospholipase D from carrot plastids occurred in the coagulum formed by the substrate and plastids in the presence of organic solvents. On the other hand Heller and Arad (122) showed that

TABLE II

HYDROLYSIS OF EGG LECITHIN, HYDROGENATED EGG LECITHIN AND DIALKYL ETHER PHOSPHOLIPIDS BY HIGH CONCENTRATION OF PHOSPHOLIPASE D

Enzyme: Type I. Standard reaction mixture A (half-quantities). Enzyme solution contained 4 mg/0.5 ml. Organic Solvent: $(C_2H_5)_2O$:$CHCl_3$ 10:1 v/v.

Substrate	Time incubated (minutes)	µmoles choline liberated	% of total substrate hydrolyzed
Egg lecithin	0	0	0
Egg lecithin	15	6.9	86
Egg lecithin	30	7.8	98
Egg lecithin	60	8.0	100
Hydrolecithin	30	2.0	25
Hydrolecithin	60	4.8	60
14C EPC	30	0.8	10
14C EPC	60	1.8	23
16C EPC	30	0.5	6
16C EPC	60	1.1	14
18C EPC	30	0.3	4
18C EPC	60	0.8	10

when peanut phospholipase D was used, the hydrolysis took place in the aqueous phase. In order to see whether the enzymic hydrolysis took place in the organic phase, in the aqueous phase or at the solvent-water interface with soluble Type I enzyme, the following experiments were carried out: The contents of a standard reaction mixture A (half-quantities) with Type I enzyme (2 mg/0.5 ml) and 0.5 ml $(C_2H_5)_2O$ were mixed vigorously for 10 seconds and then centrifuged immediately at top speed with an International Clinical Centrifuge for about 30 seconds. At this stage, three layers were observed in each tube. The top layer was the ethereal layer (saturated with water), the interfacial layer was a thick gelatinous film, and the bottom layer was the aqueous layer (saturated with diethyl ether). These layers were separated and each analyzed for enzyme activity (Table III).

Experiment I: The ethereal layer, interfacial layer and aqueous layer were separated and recombined immediately, the contents of the tube were mixed well and then incubated at 26°C for 30 minutes and for 60 minutes.

Experiment II: The ethereal layer was transferred to another tube and 0.625 ml of 0.1 M acetate buffer, pH 5.6, and 0.125 ml of 1 M $CaCl_2$ added. The contents of the tube were incubated at 26°C for 30 minutes and for 60 minutes.

Experiment III: The ethereal layer and the gelatinous film were collected together and transferred to another tube. 0.625 ml of 0.1 M acetate buffer, pH 5.6, 0.125 ml

TABLE III

THE LOCUS OF PHOSPHOLIPASE D ACTIVITY IN THE WATER - $(C_2H_5)_2O$ SYSTEM

Experiment	Time incubated (minutes)	Choline liberated (μmoles)			
		Ethereal layer	Ethereal + interfacial film	Aqueous + film	Recombined
I	30	-	-	-	5.5
	60	-	-	-	6.2
II	30	0.1	-	-	-
	60	0.2	-	-	-
III	60	-	4.1	-	-
IV	30	-	-	0.5	-
	60	-	-	0.6	-

of 1 M $CaCl_2$ were added. The contents of the tube were mixed and incubated at 26°C for 60 minutes.

Experiment IV: The aqueous layer and the gelatinous film were collected together and transferred to another tube. 0.5 ml $(C_2H_5)_2O$ was added. The contents of the tube were mixed and incubated at 26°C for 30 minutes and for 60 minutes.

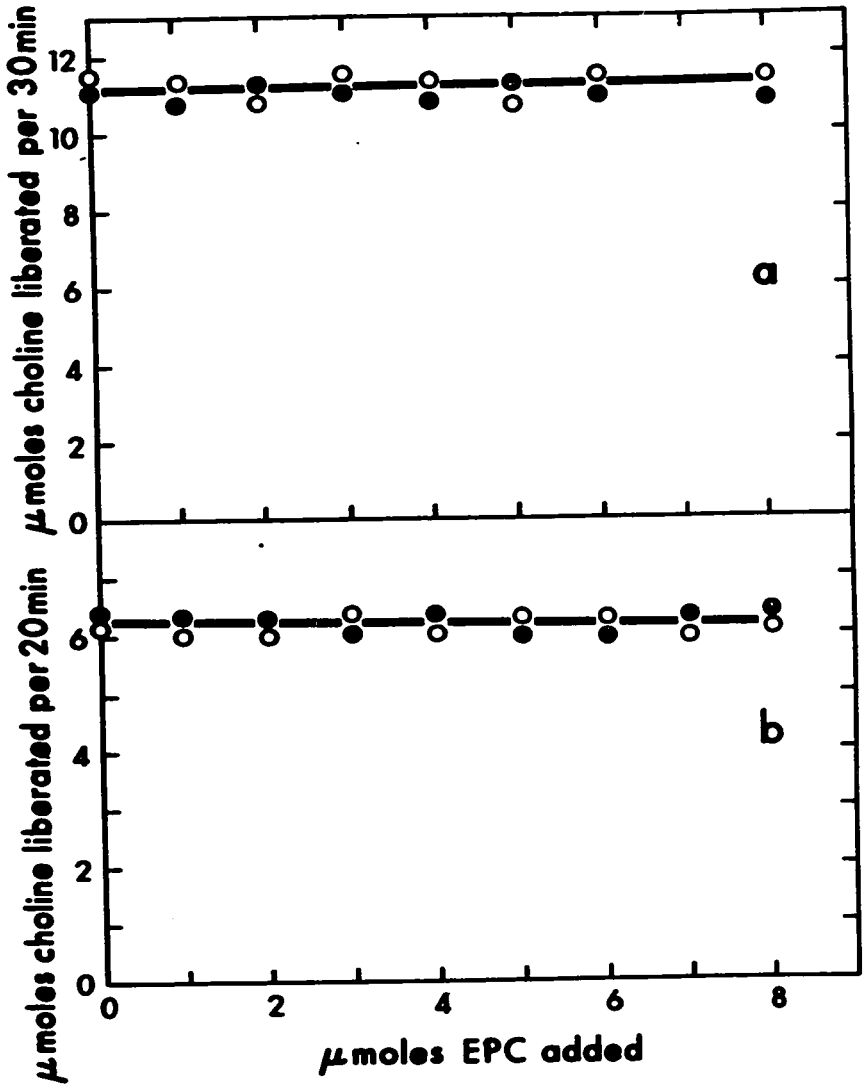
Each result shown is the average of duplicate determinations. The results indicated that the enzymic activity was mainly present in the interfacial layer. Therefore, it was of interest to analyze the film separately for its phospholipid content relative to the aqueous and ethereal layers. Therefore the partition of egg lecithin between aqueous salt solution and ethereal phase was checked under the same experimental conditions as described above. Type I enzyme (2 mg/0.5 ml) was used. It was found that 16% of the egg lecithin was in the aqueous phase, 80% in the ethereal phase and 4% in the film.

C. Effect of Dialkyl Ether Phospholipids on Egg Lecithin Hydrolysis with Organic Solvent as Activator

Since at low enzyme concentrations dialkyl ether phospholipids were not hydrolyzed (Fig. 21a and 21b), it was of interest to determine whether the dialkyl ether phospholipids affected the rate of hydrolysis of egg lecithin. A fixed amount of egg lecithin was mixed with various amounts of dialkyl ether phospholipids. Fig. 22 shows that under these conditions dialkyl ether phospholipids did not affect the hydrolysis of egg lecithin.

Figure 22a. Effect of 14C EPC and 18C EPC on the hydrolysis of egg lecithin when $(C_2H_5)_2O$:$CHCl_3$ 10:1 v/v was used as activator. Type II enzyme (1 mg/ml) and standard reaction mixture A were used. Incubated at 26°C for 30 minutes. •, 14C EPC; o, 18C EPC.

Figure 22b. Effect of 14C EPC and 18C EPC on the hydrolysis of egg lecithin when $(C_2H_5)_2O$:$CHCl_3$ 10:1 v/v was used as activator. Type I enzyme (1 mg/0.5 ml), lot no. 20C - 2090 and standard reaction mixture A (half-quantities) were used. Incubated at 26°C for 20 minutes. •, 14C EPC; o, 18C EPC.


μmoles choline liberated per 30min
12
10
8
6
4
2
0
a
μmoles choline liberated per 20min
6
4
2
0
b
0
2
4
6
8
μmoles EPC added

D. Hydrolysis of Dialkyl Ether Phospholipids using Sodium Dodecyl Sulfate as Activator

In view of the report by Dawson and Hemington (106) that SDS activated egg lecithin hydrolysis, the role of SDS was examined as activator for the hydrolysis of dialkyl ether phospholipids. It was found that the rate of hydrolysis of the dialkyl ether phospholipids with SDS as activator was much greater than when organic solvents were used. Therefore, the optimal conditions of pH, Ca^{2+} concentration and temperature were determined for hydrolysis with SDS as activator using 14 C EPC as substrate.

1. Effect of pH on the Hydrolysis of 14C EPC

The optimal pH for hydrolysis of the dialkyl ether phospholipids was between 6.5 and 7.0 when SDS was used as activator (Fig. 23). This range was similar to that for egg lecithin hydrolysis (130).

2. Effect of Ca^{2+} on the Hydrolysis of 14C EPC

In the absence of Ca^{2+}, dialkyl ether phospholipids were not hydrolyzed. The optimal Ca^{2+} concentration was 0.1 M (final concentration in reaction medium). Table IV shows the effect of Ca^{2+} on the hydrolysis of dialkyl ether phospholipids.

3. Effect of Temperature on the Hydrolysis of 14C EPC

The temperature at which maximum hydrolysis of 14C EPC was observed at 37°C (Fig. 24). This differs from that (26°C) for the hydrolysis of egg lecithin (106). The temperature at which maximum hydrolysis of 18C EPC was observed to be 37°C.

Figure 23. Effect of pH on the hydrolysis of 14C EPC when SDS was used as activator. Standard reaction mixture B, Type I enzyme (4 mg/ml), lot no. 20C - 2090 and 16 μmoles of 14C EPC were used. Incubated at 26°C for 1 hour.

●, 16 μmoles SDS; o, 20 μmoles SDS.

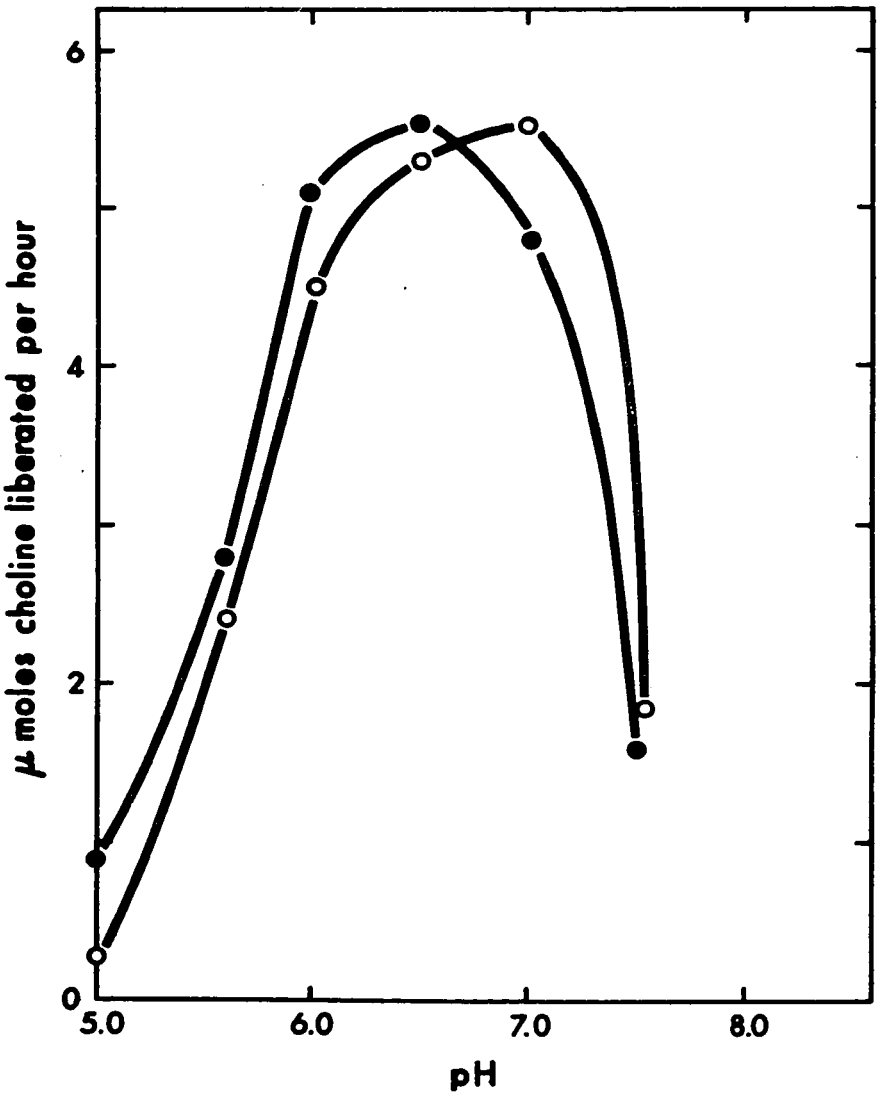

μ moles choline liberated per hour
0
2
4
6
5.0
6.0
7.0
8.0
pH

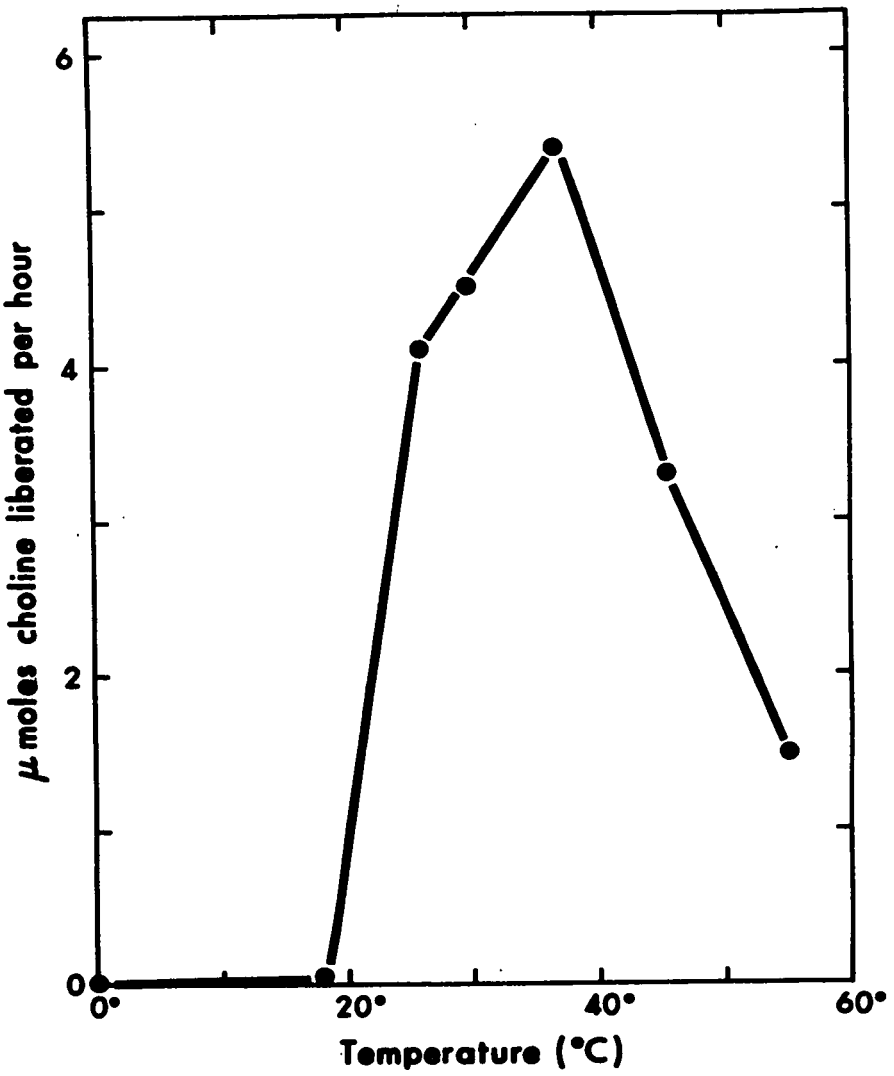
TABLE IV

EFFECT OF Ca^{2+} ON THE HYDROLYSIS OF 14C EPC BY PHOSPHOLIPASE D

Enzyme: Type I (4 mg/1.0 ml). Standard reaction mixture B, 16 µmoles of 14C EPC and 16 µmoles SDS, pH 7.0. Incubated at 37°C for one hour.

$CaCl_2$ concentration (M) (final conc.)	Choline liberated (µmoles)	% Hydrolysis
0	0	0
0.01	1.6	10
0.05	2.5	16
0.10	4.1	26
0.50	0.6	4
1.00	0.4	3

Figure 24. Effect of temperature on the hydrolysis of 14C EPC when SDS was used as activator. Standard reaction mixture B, pH 7.0, Type I enzyme (4 mg/ml), lot no. 20C - 2090, 16 µmoles of 14C EPC and 16 µmoles of SDS were used. Incubated for 1 hour.


6
4
2
0
μ moles choline liberated per hour
0°
20°
40°
60°
Temperature (°C)

4. Time Course of 14C EPC Hydrolysis

Fig. 25 shows the time course of hydrolysis of 14C EPC by phospholipase D. After the reaction had proceeded for one hour, the rate of hydrolysis had greatly decreased from the initial value probably due largely to denaturation of enzyme in the presence of SDS at 37°C.

5. Effect of Substrate Concentration on the Hydrolysis of 14C EPC at Constant SDS Concentration

Fig. 26 shows the effect of substrate concentration on the hydrolysis rate. When 20 μmoles SDS were used, optimal hydrolysis was observed with 20 μmoles 14C EPC. This figure may reflect an optimal 1:1 molar ratio of SDS:14C EPC. Therefore, the effect of SDS concentration on the hydrolysis of 14C EPC at fixed 14C EPC concentration was examined.

6. Effect of SDS Concentration on the Hydrolysis of Dialkyl Ether Phospholipids at Fixed Substrate Concentration

We first confirmed that the optimal hydrolysis of egg lecithin was obtained with a molar ratio SDS:lecithin of 1:2 (106). For the hydrolysis of dialkyl ether phospholipids, higher SDS:dialkyl ether phospholipids molar ratios (approximately 1:1) were found to be optimal. The results are shown in Fig. 27.

7. Effect of Enzyme Concentration on the Hydrolysis of Dialkyl Ether Phospholipids

Since at low enzyme concentrations, dialkyl ether phospholipids were poorly hydrolyzed, we examined the effect of enzyme concentration on the extent of hydrolysis after one hour. The results are shown in Fig. 28. It can be seen that the rates of hydrolysis were 14C EPC 16C EPC 18C EPC over the range of enzyme concentration examined.

Figure 25. Hydrolysis of 14C EPC by phospholipase D. Standard reaction mixture B, pH 7.0, Type I enzyme (4 mg/ml), lot no. 20C - 2090, 16 μmoles of 14C EPC and 16 μmoles SDS were used. Incubated at 37°C.

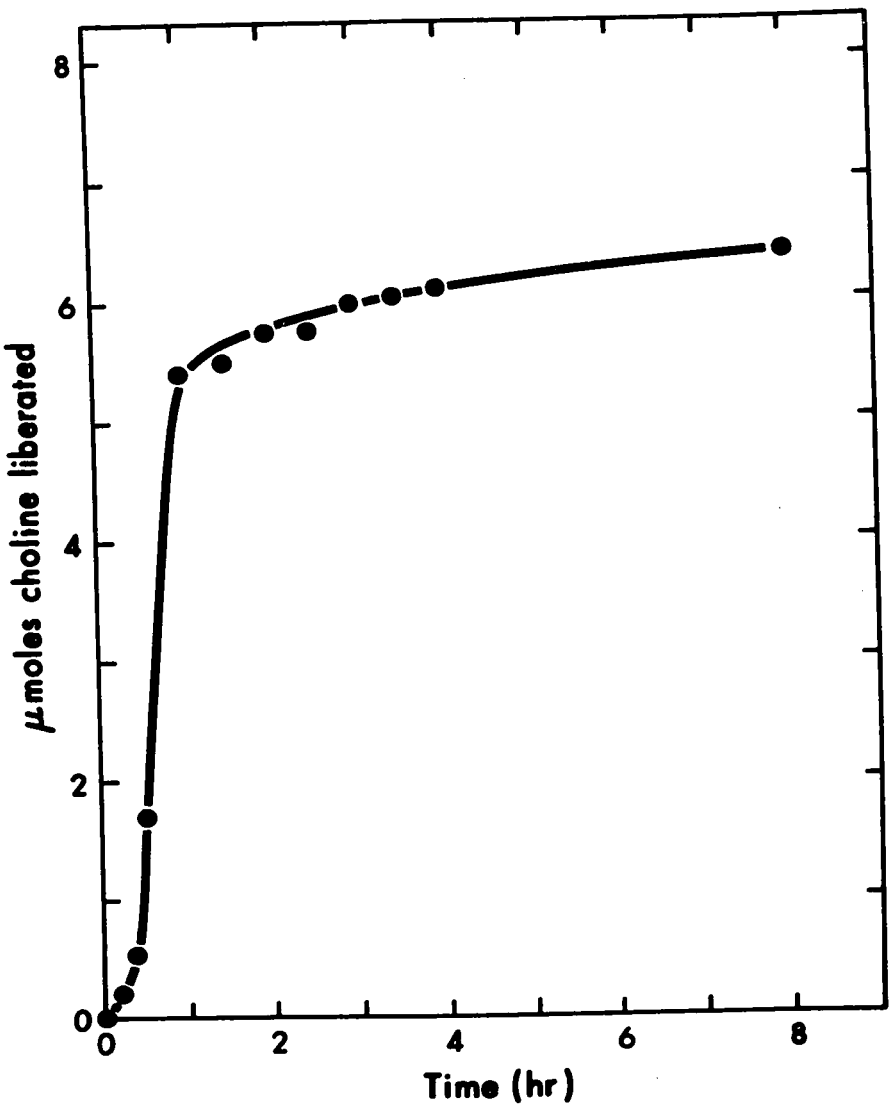

8
6
4
2
0
0
2
4
6
8
μmoles choline liberated
Time (hr)

Figure 26. Effect of substrate concentration on the hydrolysis of 14C EPC. Standard reaction mixture B, pH 7.0, Type I enzyme (4 mg/ml), lot no. 20C - 2090 and 20 µmoles SDS were used. Incubated at 37°C for 1 hour.

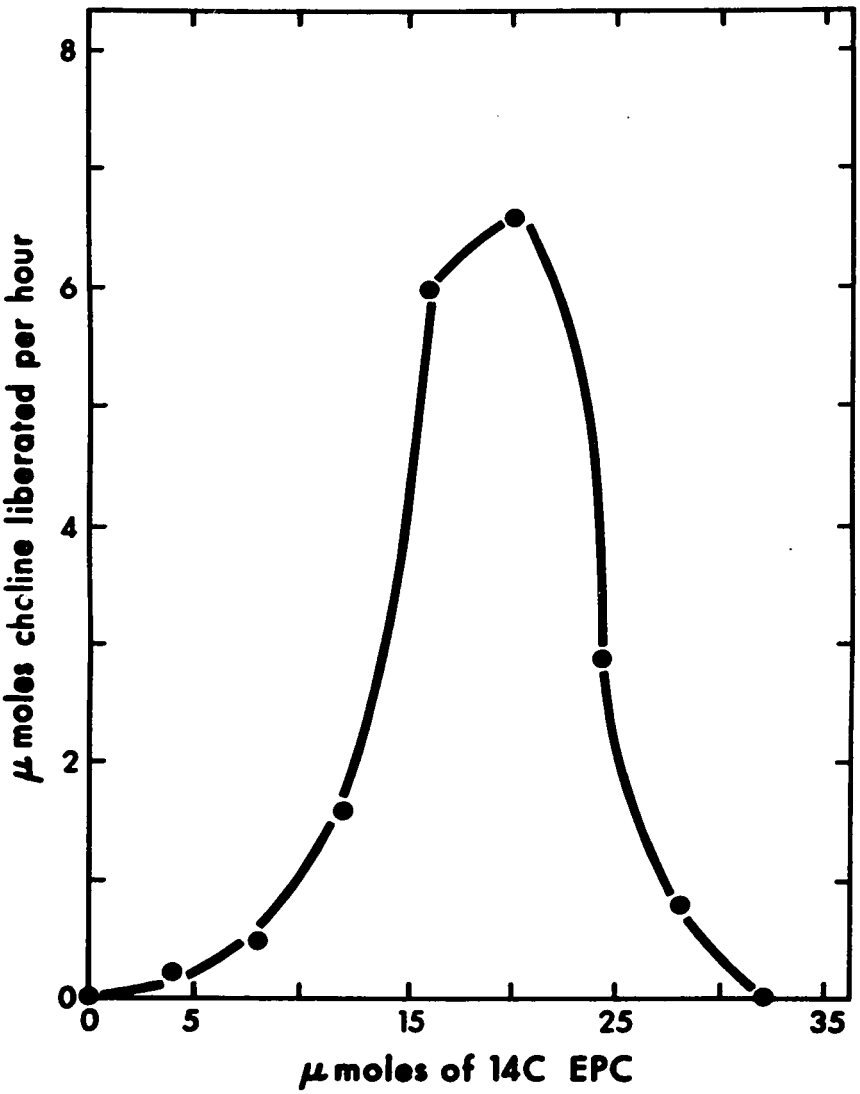

8
6
4
2
0
0
5
15
25
35
μ moles chcline liberated per hour
μ moles of 14C EPC

Figure 27. Effect of SDS on the hydrolysis of dialkyl ether phospholipids. Standard reaction mixture B, pH 7.0. Type I enzyme (4 mg/ml), lot no. 110C - 1490, and 16 μmoles of EPC were used. Incubated at 37°C for 1 hour. ●, 14C EPC; ■, 16C EPC; ▲, 18C EPC.

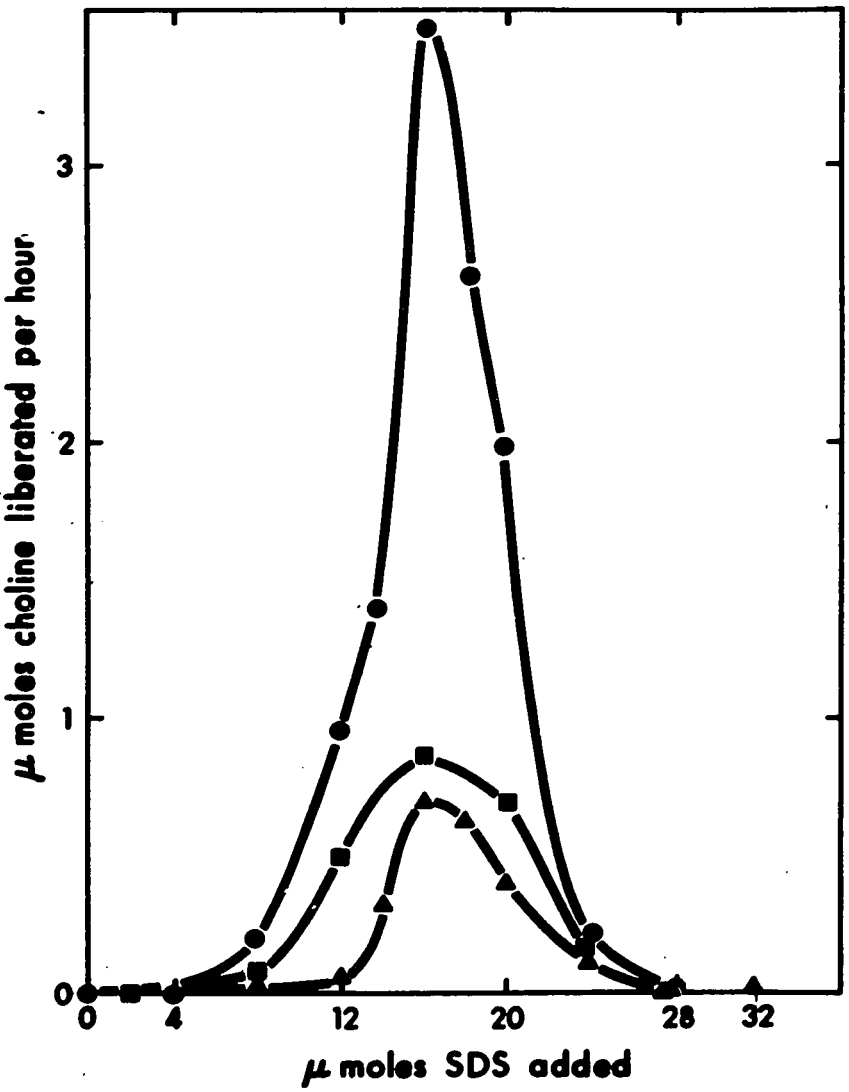
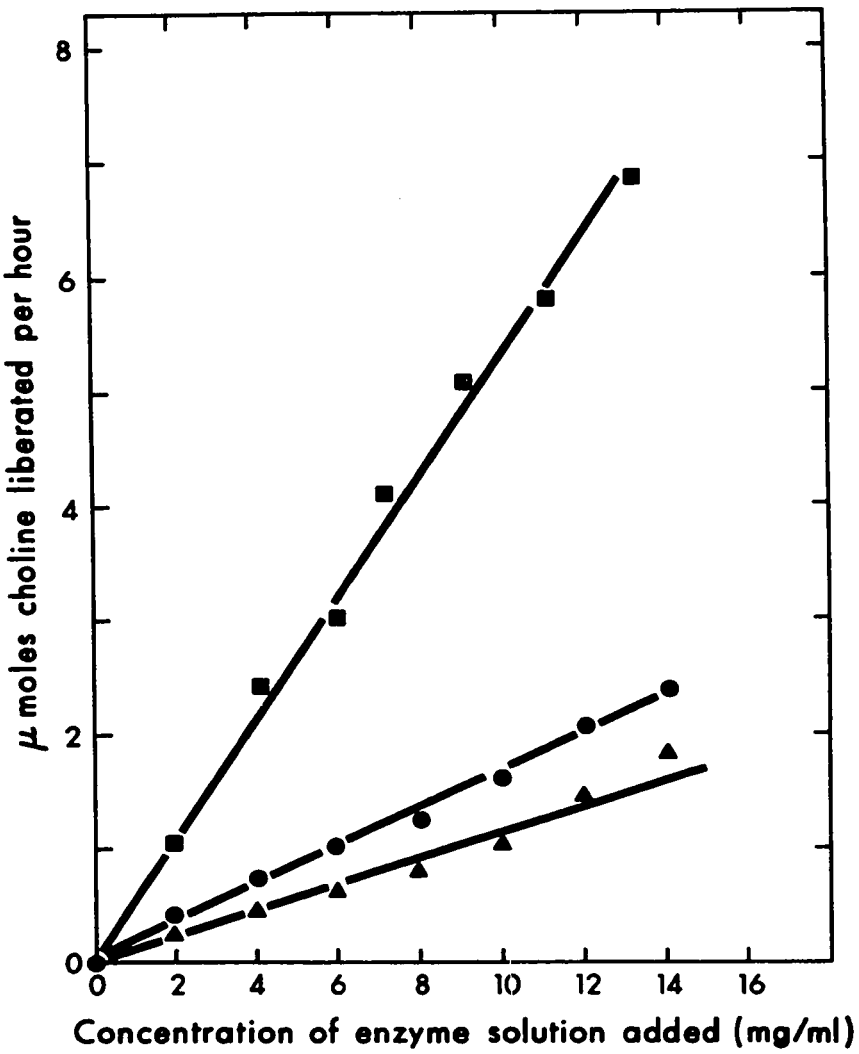

μ moles choline liberated per hour
3
2
1
0
0
4
12
20
28
32
μ moles SDS added

Figure 28. Effect of enzyme concentration on the hydrolysis of dialkyl ether phospholipids. Standard reaction mixture B, pH 7.0, Type I enzyme (lot no. 110C - 1490), 16 μmoles EPC and 16 μmoles SDS were used. Incubated at 37°C for 1 hour. ■, 14C EPC; ●, 16C EPC; ▲, 18C EPC.


μ moles choline liberated per hour
0
2
4
6
8
0
2
4
6
8
10
12
14
16
Concentration of enzyme solution added (mg/ml)

E. Turbidimetric Studies of Phospholipid: Detergent Systems

It was observed visually that at a given SDS:phospholipid ratio, different turbidities were obtained with different phospholipids. For example, 18C EPC gave much more turbid preparations than did 14C EPC. This was examined quantitatively by measuring the optical densities at 500 nm (Fig. 29). All the dialkyl ether phospholipid dispersions were progressively cleared by increasing amounts of SDS and the higher homologs required more SDS to produce a given decrement in absorbance. Similar results were obtained when cetyltrimethylammonium bromide (CTABr) and polyoxyethylene cetyl ether (BRIJ 58) were used to disperse the dialkyl ether phospholipids (Fig. 30 and Fig. 31).

F. Binding of Enzyme to Substrates in the Presence of SDS

It was reported (106) that SDS enhanced the binding of phospholipase D to egg lecithin. The lower rates of hydrolysis of dialkyl ether phospholipids as compared with the diacyl phospholipids could have been due to differences in the ability of these substrates to bind the enzyme. We carried out experiments to test whether the enzyme was adsorbed onto the dialkyl ether phospholipids in the presence of Ca^{2+} and SDS. The procedure used was similar to that already reported (106).

Egg lecithin or 14C EPC (16 μmoles) was dispersed in 1.25 ml of 0.1 M buffer solution, pH 6.5, and 4 μmoles of SDS and 0.25 ml of 1 M $CaCl_2$ were added. The mixtures were kept at 0°C for several minutes. 1 ml of enzyme solution containing 2 mg of Type I enzyme was added and the mixtures were shaken for 2 to 3 minutes at 0°C. The mixtures were centrifuged at 100,000 g (S.W. 50.1 rotor, Beckman-Spinco Model L-265B) at 0°C for 30 minutes. The precipitate and

Figure 29. Effect of SDS on the optical density of phospholipid-water preparations. Each phospholipid (32 μmoles) was dispersed in 2.5 ml of 0.1 M buffer, pH 6.5, in a jacketed glass vessel. The temperature was maintained by circulating water at 26°C. SDS was added and mixed with a magnetic stirring bar. Aliquots of 0.1 ml were withdrawn into 1.9 ml of distilled water and the optical density was measured at 500 nm. ●, 14C EPC; ▲, 18C EPC; ■, hydrogenated lecithin.

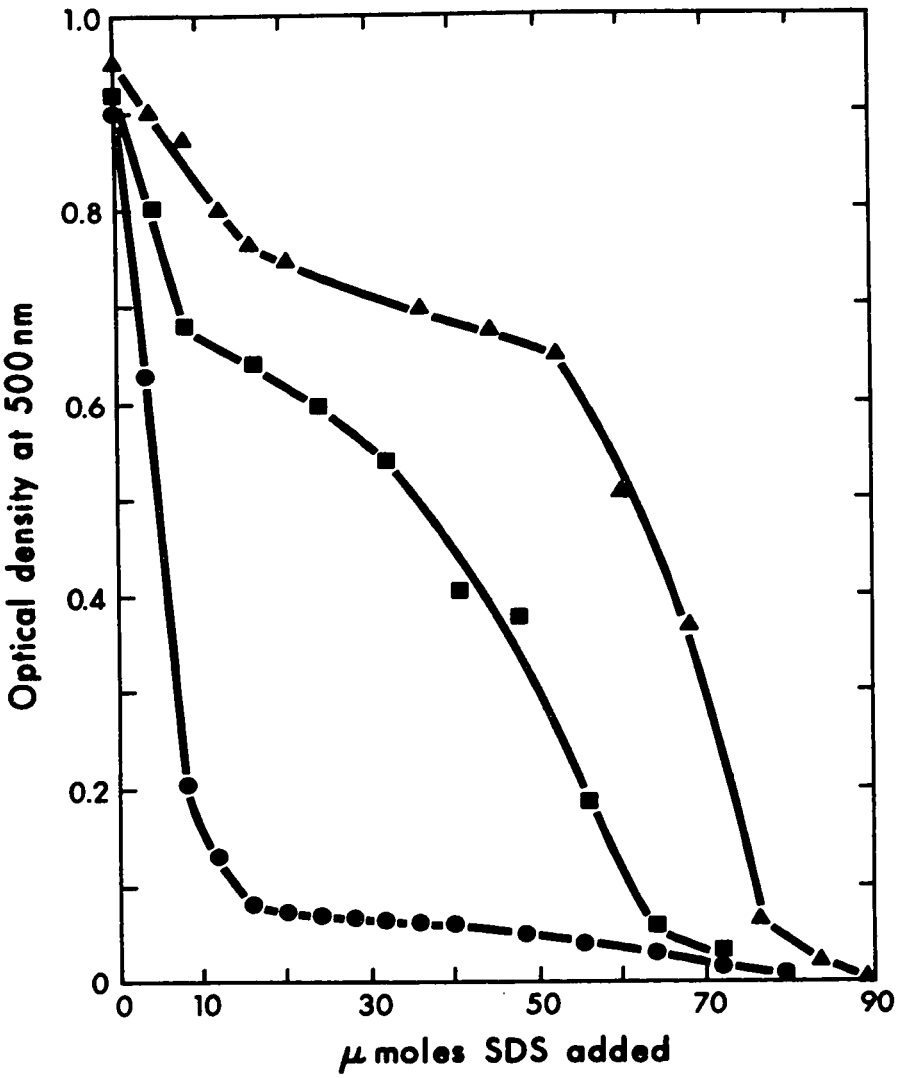

1.0
0.8
0.6
0.4
0.2
0
Optical density at 500nm
0
10
30
50
70
90
μ moles SDS added

Figure 30. Effect of CTABr on the optical density of dialkyl ether phospholipid-water preparations. Same experimental details as Figure 29.

●, 14C EPC; o, 16C EPC.

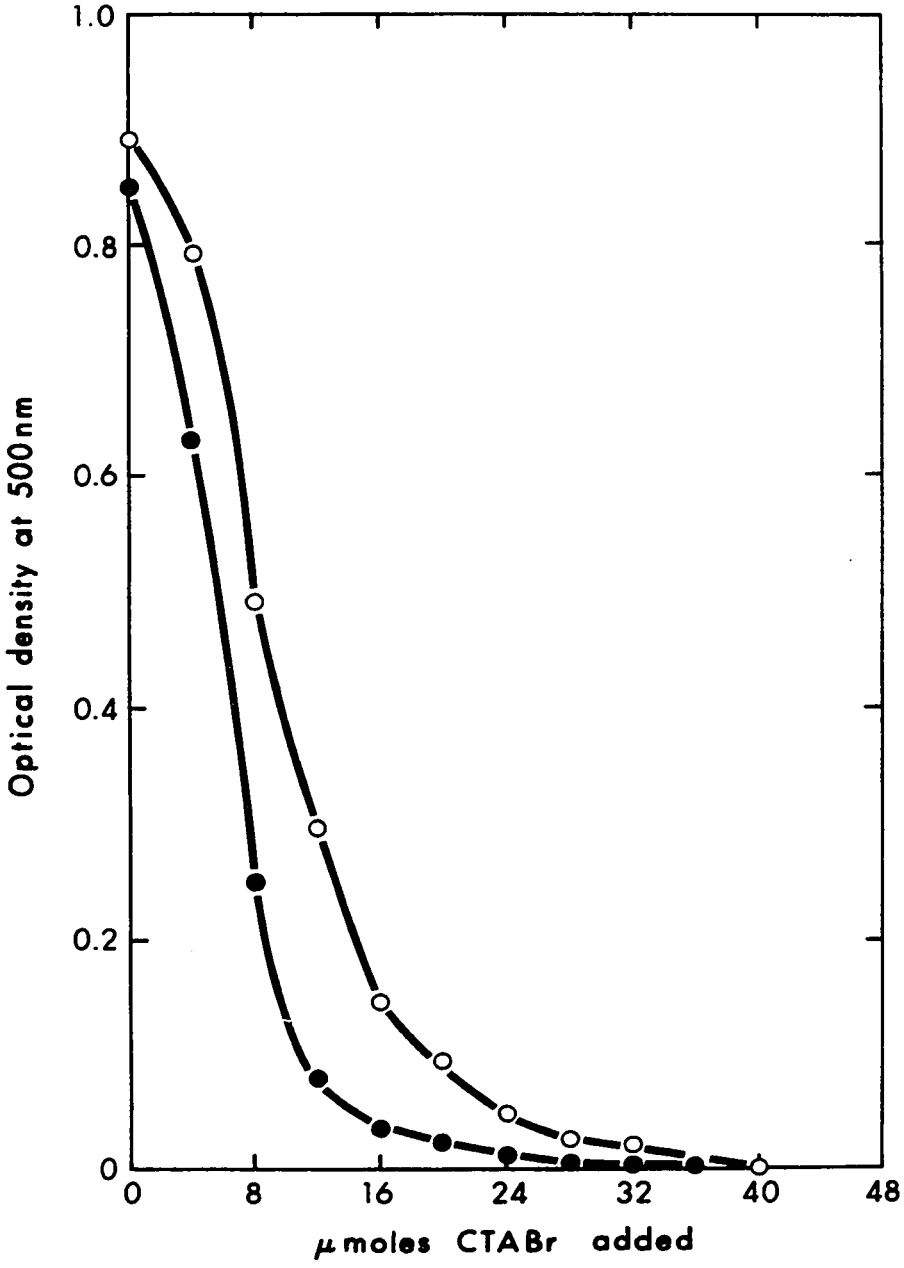
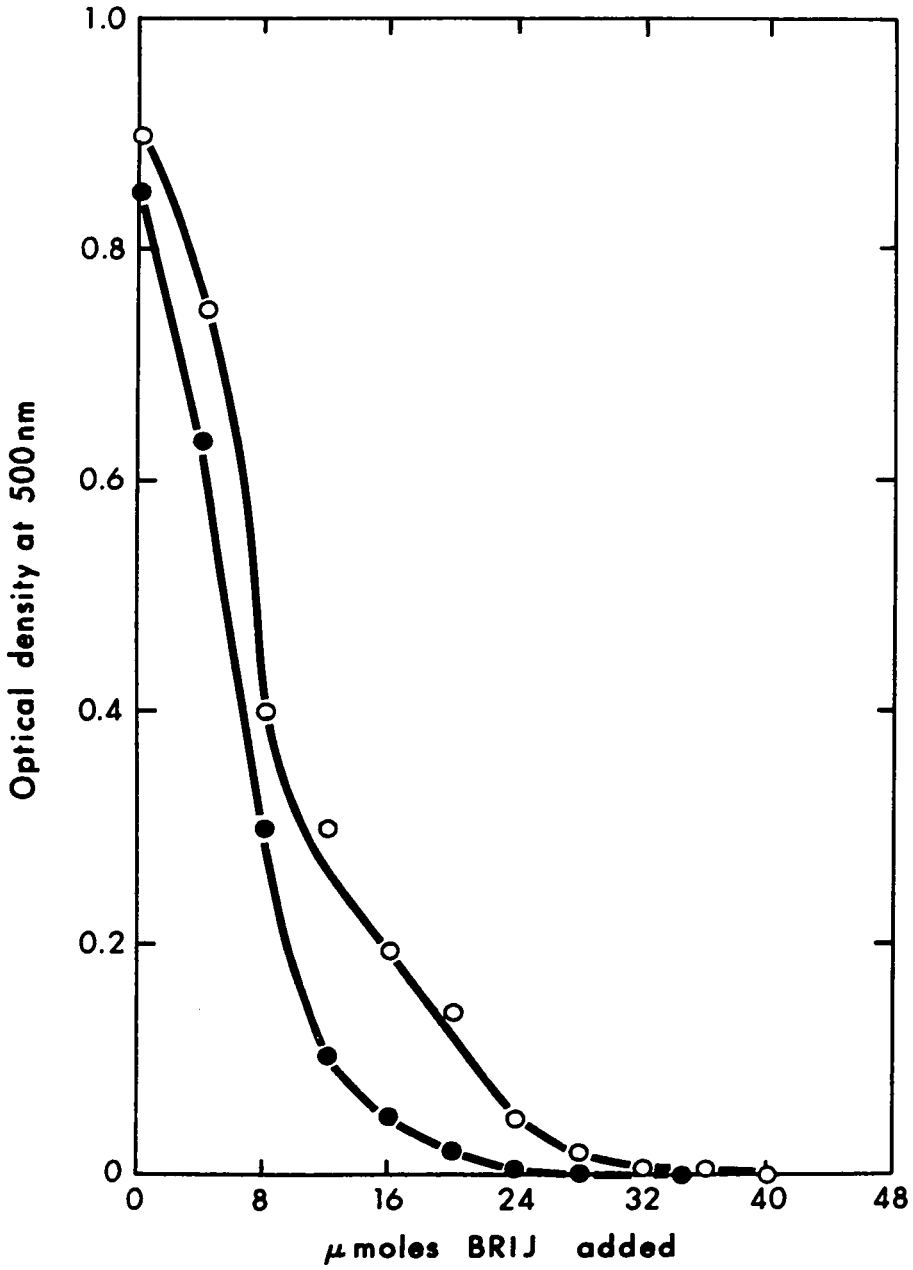

1.0
0.8
0.6
0.4
0.2
0
Optical density at 500nm
0
8
16
24
32
40
48
μ moles CTABr added

Figure 31. Effect of BRIJ 58 on the optical density of dialkyl ether phospholipid-water preparations. Same experimental details as Figure 29.

●, 14C EPC; o, 16C EPC.


1.0
0.8
0.6
0.4
0.2
0
Optical density at 500nm
0
8
16
24
32
40
48
μ moles BRIJ added

supernatant were assayed for enzymic activity as follows:

I. The sediment from the tube containing 14C EPC was dispersed in 1 ml of water and transferred to another tube containing 16 μmoles egg lecithin, 1.25 ml of 0.1 M buffer solution, pH 6.5. 0.25 ml of 1 M $CaCl_2$ and 4 μmoles SDS. The mixtures were mixed and incubated at 26°C for 30 minutes.

II. The supernatant from the 14C EPC tube was added to another tube containing 16 μmoles egg lecithin and 4 μmoles SDS mixed and incubated as above.

III. The sediment from the tube containing egg lecithin was dispersed in 1 ml of water and added to a tube containing 1.25 ml of 0.1 M buffer solution, pH 6.5, 0.25 ml of 1 M $CaCl_2$ and 4 μmoles SDS. The mixtures were incubated as above.

IV. The supernatant of the tube containing egg lecithin was added to a tube containing 16 μmoles egg lecithin and 4 μmoles SDS, mixed and incubated as above.

Table V shows the results of these experiments. The results suggested that a considerable proportion of the enzyme was adsorbed onto egg lecithin. Under the same conditions, no enzyme appeared to be adsorbed onto 14C EPC and all the enzyme activity remained in the supernatant.

TABLE V

BINDING OF ENZYME TO SUBSTRATES IN THE PRESENCE OF Ca^{2+} AND SDS

Expt. No.	Lipid mixture			
	PC + SDS		EPC + SDS	
	Choline liberated (μmoles)		Choline liberated (μmoles)	
	supernatant	sediment	supernatant	sediment
1	2.30	4.75	6.0	0
2	2.70	-	6.0	0

G. Effect of Cetyltrimethylammonium Bromide (CTABr) on the Hydrolysis of Dialkyl Ether Phospholipids

Although a cationic amphipathic compound, cetyltrimethylammonium bromide (CTABr), was able to disperse dialkyl ether phospholipids (Fig. 30), it was found to be a potent inhibitor of phospholipase D action (Fig. 32). When 14C EPC was first mixed with an amount of SDS optimal for hydrolysis (14C EPC:SDS = 1:1 molar ratio), subsequent addition of CTABr also inhibited the hydrolytic reaction (Fig. 32). However, when an excess amount of SDS was initially present (14C EPC:SDS = 1:1.5 molar ratio), such that the system was inhibited by the excess of anionic detergent, then addition of CTABr reversed this inhibition (Fig. 32).

H. Effect of Nonionic Detergent (BRIJ 58) on the Hydrolysis of Egg-lecithin and Dialkyl Ether Phospholipid

Since nonionic detergent, such as BRIJ 58, could disperse dialkyl ether phospholipids (Fig. 31), it was of interest to determine the effect of this nonionic detergent on the phospholipase D action. Table VI and VII show that BRIJ 58 can activate the hydrolysis of egg-lecithin and 14C EPC.

I. Effect of Phosphatidic Acids on the Hydrolysis of Egg-lecithin and Dialkyl Ether Phospholipids

From the work of Dawson and Hemington (106, 130) and from results described in this chapter and in Chapter V, it seemed possible that the enzymic activity might be controlled by two factors: (a) a surface charge requirement of the substrate, (b) the transition temperature for gel-liquid crystalline structure of the substrate. On

Figure 32. Effect of CTABr on the hydrolysis of 14C EPC. Standard reaction mixture B (half-quantities), pH 6.5, Type I enzyme (4 mg/0.5 ml), lot no. 110 - 1490 and 8 µmoles 14C I-P were used. Incubated at 37°C for 1 hour. o, hand-shaken dispersion of 14C EPC (8 µmoles); •, hand-shaken dispersion of 14C EPC (8 µmoles) with sodium dodecyl sulfate (8 µmoles) added; ■, hand-shaken dispersion of 14C EPC (8 µmoles) with sodium dodecyl sulfate (12 µmoles) added.

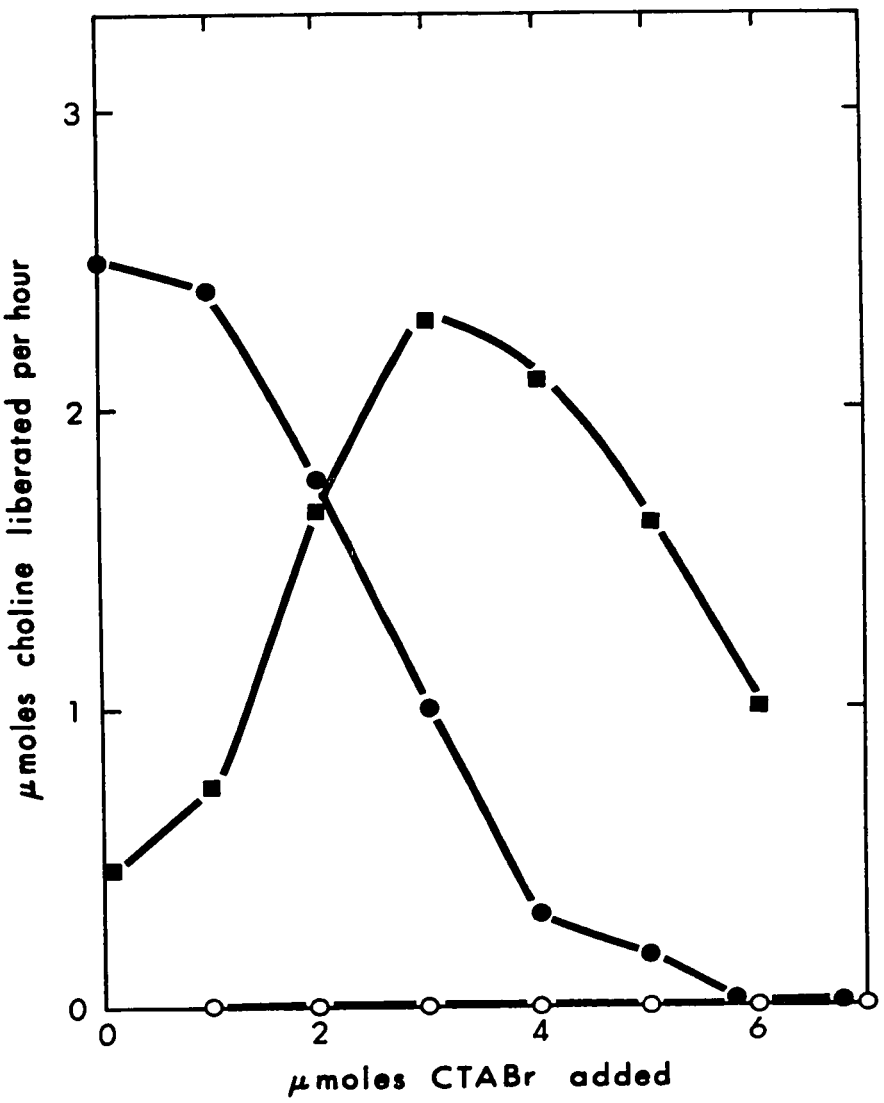

3
2
1
0
0
2
4
6
μmoles choline liberated per hour
μmoles CTABr added

TABLE VI

EFFECT OF NONIONIC DETERGENT (BRIJ 58) ON THE HYDROLYSIS OF EGG-YOLK LECITHIN BY PHOSPHOLIPASE D

Standard reaction mixture B, (half quantities), pH 5.6, Type I enzyme (4 mg/0.5 ml), lot no. 110C - 1490 and 8 µmoles egg-yolk lecithin were used. Incubated at 26°C for 10 minutes. The SDS (4 µmoles) control experiment was done under the same condition except that pH 6.5 buffer solution was used.

µmoles BRIJ added added	µmoles choline liberated in 10 minutes	Relative activation % SDS control
0	0	0
1	0.5	7
2	1.1	15
3	3.0	43
4	4.6	66
5	5.7	80
6	5.7	80
8	5.6	80
10	5.6	80

TABLE VII

EFFECT OF NONIONIC DETERGENT (BRIJ 58) ON THE HYDROLYSIS OF 14C EPC BY PHOSPHOLIPASE D

Standard reaction mixture (half quantities), pH 5.6, Type I enzyme (4 mg/0.5 ml), lot no. 110C - 1490 and 8 µmoles 14C EPC were used. Incubated at 37°C for 1 hour. The SDS (8 µmoles) control experiment was done under the same condition except that pH 6.5 buffer was used.

µmoles BRIJ added	µmoles choline liberated in 10 minutes	Relative activation % SDS control
0	0	0
1	0	0
2	0.2	7
3	0.5	17
4	1.0	33
5	1.8	60
6	2.4	80
8	2.4	80
10	2.3	77

this basis, it was possible to make rational predictions of rate effects on mixing various phosphatidic acids with egg-lecithin or dialkyl ether phospholipids. The data shown in Figs. 33-35 were obtained to test these predictions.

Fig. 33 shows the effect of egg Na_2PA (upper figure) and 14C EPA and 14C Na_2EPA on the hydrolysis of egg lecithin. Egg Na_2PA was clearly a more powerful activator than 14C Na_2EPA. When 14C EPA was used, it activated hydrolysis at low concentrations but not at high concentrations (optimum 4 μmoles 14C EPA when 8 μmoles egg lecithin was used). 16C EPA and 18C EPA gave similar results but were slightly less effective.

Fig. 34 shows that low concentrations of 14C EPA activated the hydrolysis of 14C EPC whereas higher concentrations were decreasingly effective (optimum 6 μmoles 14C EPA when 8 μmoles 14C EPC were used). When 4 μmoles SDS was initially present with the substrate, 14C EPA was much more effective as an activator (optimum 4 μmoles 14C EPA).

Fig. 35 shows the effects of egg Na_2PA and 14C EPA on the hydrolysis of 14C EPC and the effects of egg Na_2PA and 16C EPA on the hydrolysis of 16C EPC. Both 14C EPA and egg Na_2PA were able to activate the hydrolysis of 14C EPC; the graphs in each case were again found to be characteristic for acid and sodium salt respectively. Maximum stimulation of hydrolysis was obtained with 6μmoles Na_2PA. In contrast, 16C EPA was unable to activate hydrolysis of 16C EPC. A critical amount (6 μmoles) of egg Na_2PA was required to activate the hydrolysis of 16C EPC. Maximum stimulation was obtained with 12 μmoles Na_2PA.

Figure 33a. Effect of Na_2PA on the hydrolysis of egg lecithin. Standard mixture B (half-quantities), pH 6.5, Type I enzyme (2 mg/0.5 ml), lot no. 20C - 2090 and 8 μmoles of egg lecithin were used. Incubated at 26°C for 1 hour.

Figure 33b. Effect of 14C EPA and 14C Na_2PA on the hydrolysis of egg lecithin. Reaction system: same as that in Figure 33a. ▲, 14C Na_2EPA; ■, 14C EPA.

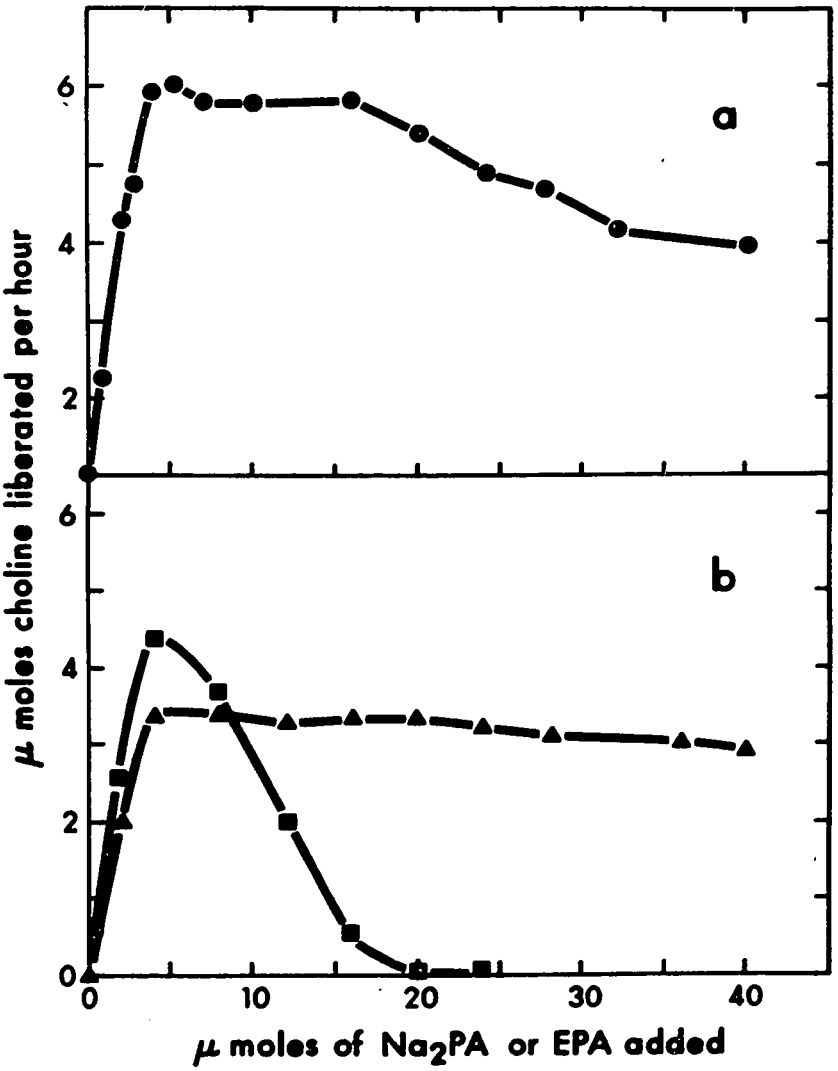

a
b
6
4
2
0
6
4
2
0
0
10
20
30
40
μ moles choline liberated per hour
μ moles of Na2PA or EPA added

Figure 34. Effect of 14C EPA on the hydrolysis of 14C EPC. Standard reaction mixture B (half quantities), Type I enzyme (2 mg/0.5 ml), lot no. 20C - 2090, and 8 μmoles of 14C EPC were used. Incubated at 37°C for 1 hour. ●, without addition of SDS, pH 6.5; ▲ with 4 μmoles SDS, pH 7.5.

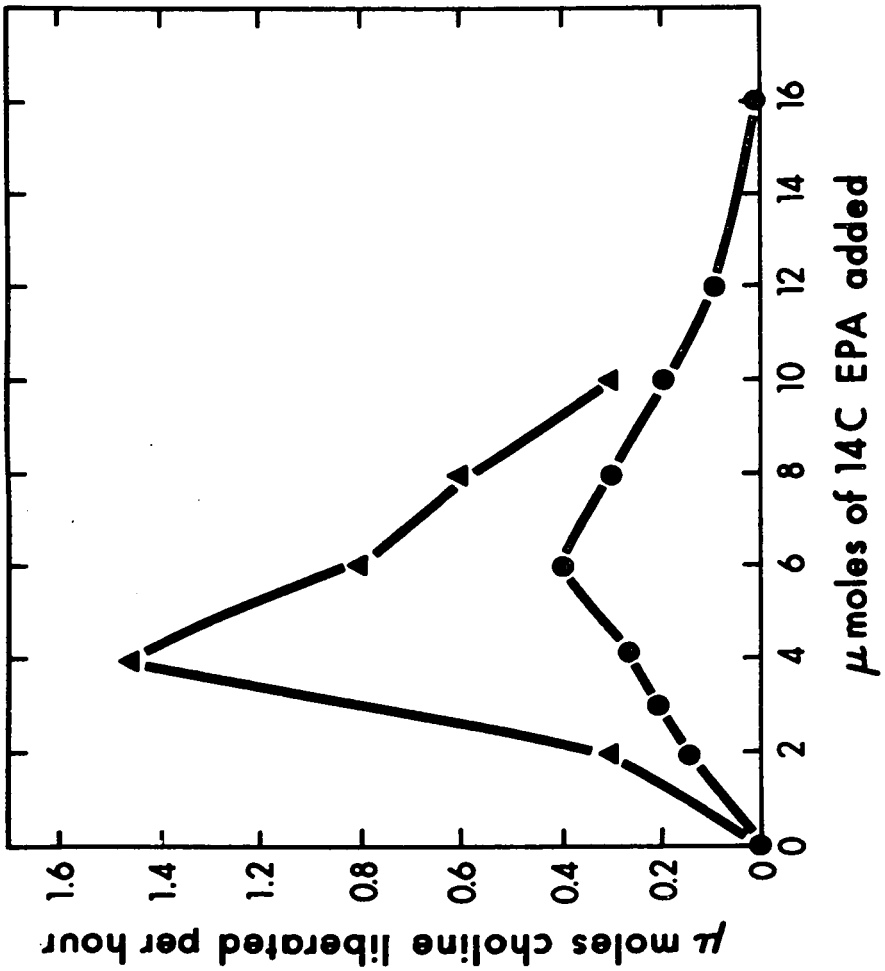
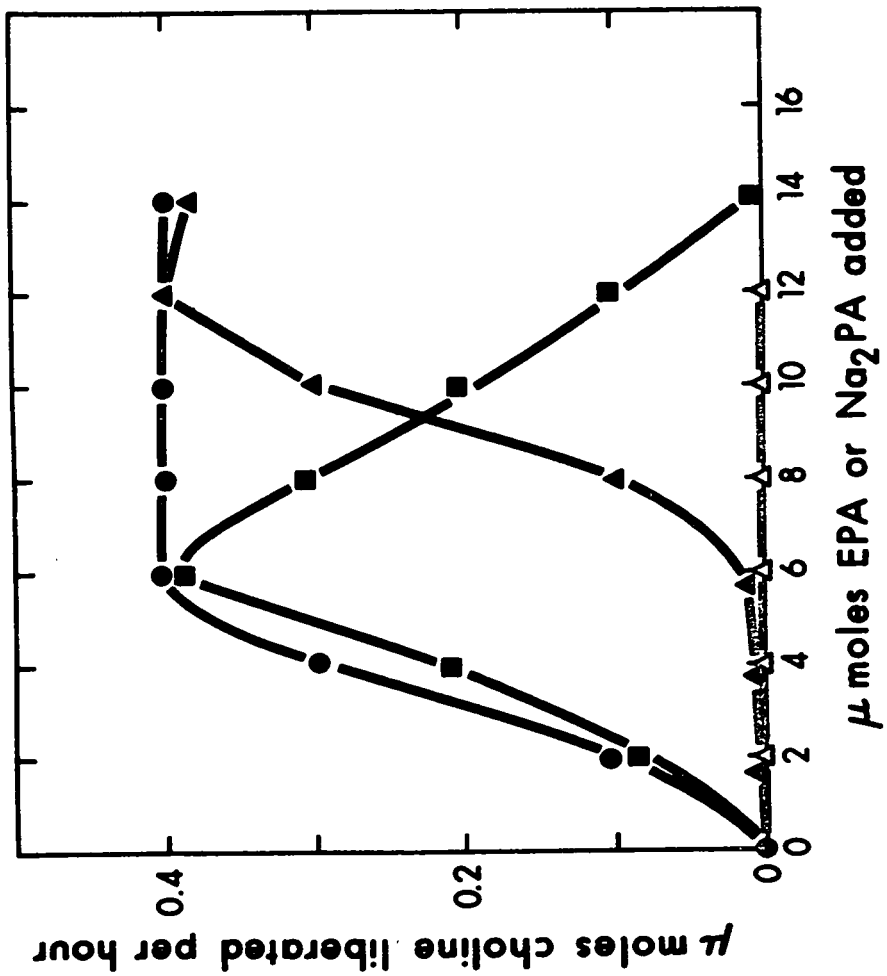

μ moles choline liberated per hour
1.6
1.4
1.2
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
μ moles of 14C EPA added

Figure 35. Effect of phosphatidic acid analogs on the hydrolysis of 14C EPC and 16C EPC. Standard reaction mixture B (half quantities), pH 6.5, Type I enzyme (2 mg/0.5 ml), lot no. 110C - 1490 and 8 μmoles EPC were used. Incubated at 37°C for 1 hour. ●, 14C EPC + Na_2PA; ■, 14C EPC + 14C EPA; ▲, 16C EPC + Na_2PA; Δ, 16C EPC + 16C EPA.


μ moles choline liberated per hour
0.4
0.2
0
0
2
4
6
8
10
12
14
16
μ moles EPA or Na2PA added

J. Effect of Dialkyl Ether Phospholipids on the Hydrolysis of Egg-yolk Lecithin when SDS was used as Activator

Since it had been found that the dialkyl ether phospholipids required more SDS than the diacyl compounds to obtain optimal reaction rates, the effects of 14C, 16C and 18C EPC on the hydrolysis of egg lecithin at a fixed SDS concentration were determined. The dialkyl ether phospholipids were found to be quite inhibitory (Fig. 36) in marked contrast with the results which had been obtained when organic solvents were used as activators (Fig. 23). Complete inhibition was obtained when 4 μmoles of any dialkyl ether phospholipid was added to 8 μmoles of egg lecithin, with 4 μmoles SDS. However, if at this point or at higher levels of ether phospholipids, additional SDS were added, the reaction rate was restored indicating that the effect of the dialkyl ether phospholipids was exerted through competition with the substrate for SDS.

K. Hydrolysis of (Choline) Plasmalogen by Cabbage Phospholipase D using Organic Solvent as Activator

In the absence of organic solvent, the choline plasmalogen was not hydrolyzed by phospholipase D. In the presence of $(C_2H_5)_2O$ - $CHCl_3$ (10:1 v/v), it was slowly hydrolyzed. Fig. 37 shows the time course of hydrolysis for choline plasmalogen and 14C EPC under these conditions. After 8 hours, only 30% of the total choline plasmalogen and 25% of the total 14C EPC were hydrolyzed. However, under the same conditions, 92% of the total egg-lecithin was hydrolyzed in 30 minutes.

Figure 36. Effect of EPC on the hydrolysis of egg lecithin when SDS was used as activator. Standard reaction mixture B (half quantities), type I enzyme (1 mg/0.5 ml) lot no. 20C - 2090, 8 μmoles of egg lecithin and 4 μmoles SDS were used. Incubated at 26°C for 20 minutes. o, 14C EPC; Δ, 16C EPC; ■, 18C EPC; o, 14C EPC with additional SDS used as indicated by the arrows; ▲, 16C EPC with additional SDS used as indicated by the arrows.

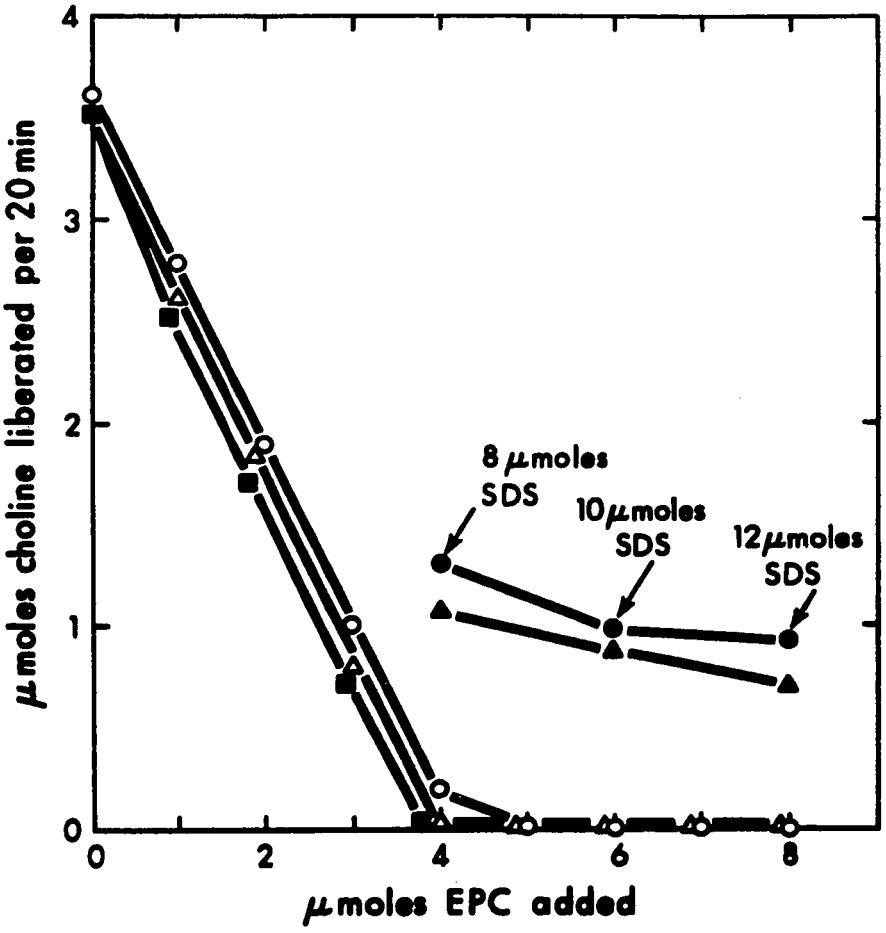
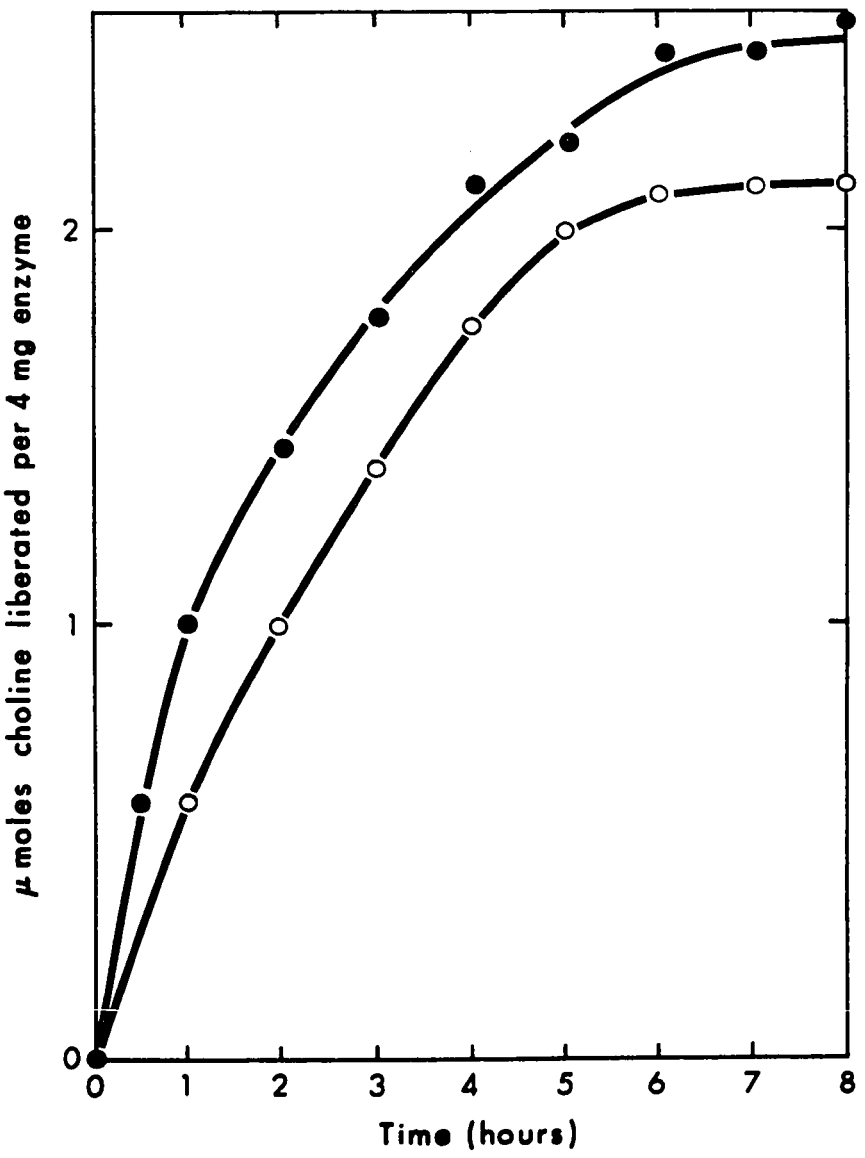

4
3
2
1
0
0
2
4
6
8
μmoles choline liberated per 20 min
μmoles EPC added
8 μmoles SDS
10 μmoles SDS
12 μmoles SDS

Figure 37. Hydrolysis of (choline) plasmalogen and 14C EPC by phospholipase D. Standard reaction mixture A (half quantity), pH 5.6, Type I enzyme (4 mg/0.5 ml), lot no. 110C - 1490 and 8 μmoles of substrates were used. Incubated at 26°C.
●, (choline) plasmalogen; o, 14C EPC.


μ moles choline liberated per 4 mg enzyme
0
1
2
0
1
2
3
4
5
6
7
8
Time (hours)

L. Hydrolysis of (Choline) Plasmalogen by Cabbage Phospholipase D using SDS as Activator

It was found that the rate of hydrolysis of choline plasmalogen with SDS as activator was much greater than when organic solvents were used. The optimal molar ratio of choline plasmalogen to SDS was about 2:1. The results are shown in Fig. 38.

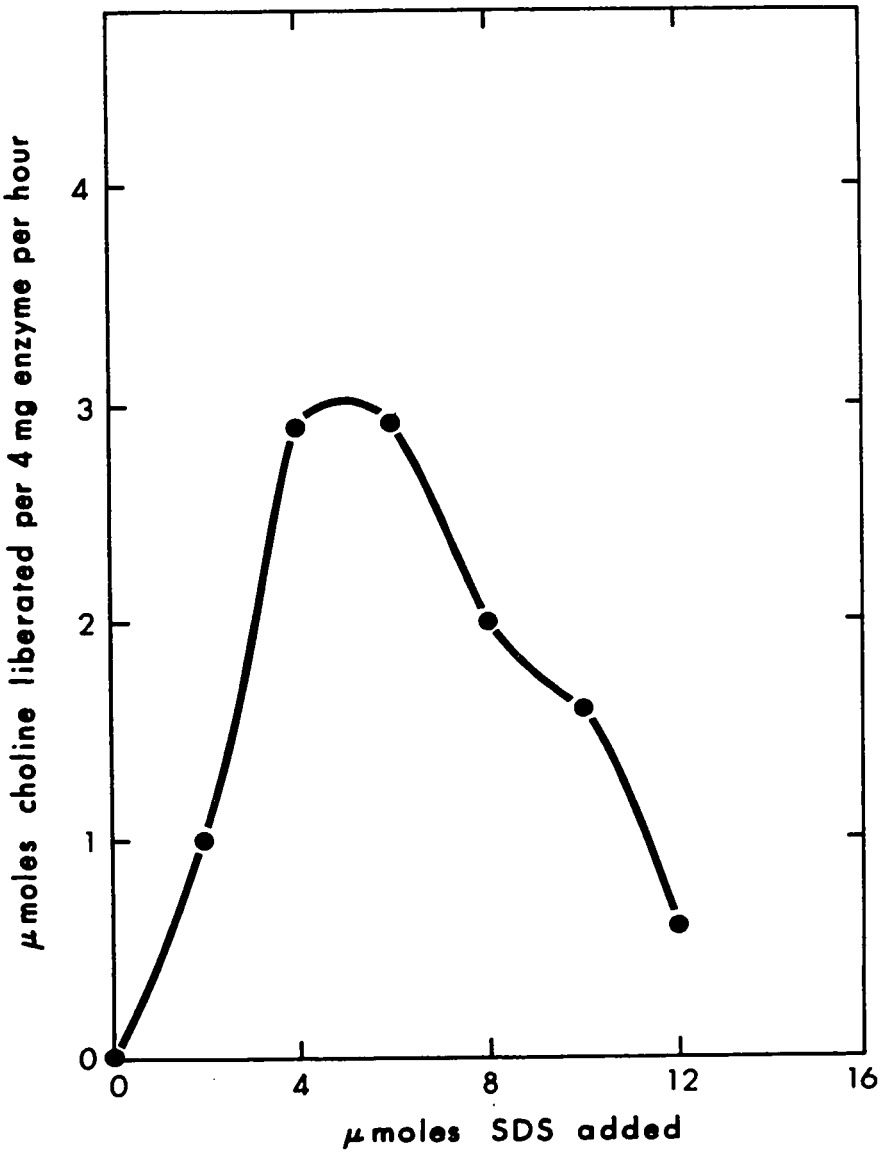
M. Hydrolysis of Sphingomyelin by Cabbage Phospholipase D using Organic Solvent as Activator

Sphingomyelin was found to be very poorly soluble in either diethyl ether or chloroform. When $(C_2H_5)_2O$-$CHCl_3$ (10:1 v/v) was used as activator and standard reaction mixture A (half quantity, 4 mg enzyme/0.5 ml) was incubated at 27°C, only 2.5% of the total substrate was hydrolyzed after 1 hour. However, under the same conditions, 92% of the egg lecithin was hydrolyzed in 30 minutes. Davidson and Long (112) also reported that sphingomyelin was slowly attacked under this condition.

N. Hydrolysis of Sphingomyelin by Cabbage Phospholipase D using SDS as Activator

In the presence of various amounts of SDS (0 - 10 μmoles), sphingomyelin (8 μmoles) was not hydrolyzed by the cabbage phospholipase D. This is in marked contrast with the results which had been obtained when egg-lecithin, dialkyl ether phospholipids or choline plasmalogen were used as substrates.

Figure 38. Effect of SDS on the hydrolysis of (choline) plasmalogen. Standard reaction mixture B (half quantity), pH 6.5. Type I enzyme (4 mg/0.5 ml), lot no. 110C - 1490 and 8 μmoles of substrate were used. Incubated at 26°C for 1 hour.


μmoles choline liberated per 4 mg enzyme per hour
0
1
2
3
4
0
4
8
12
16
μmoles SDS added

0. Discussion

The results of this study have confirmed many of the general characteristics of cabbage leaf phospholipase D activity elucidated by previous workers (106, 112, 123, 130) with respect to pH optimum, Ca^{2+} requirement and activation by anionic amphipaths and organic solvents. Other enzymes catalyzing the same hydrolytic reaction but derived from other sources exhibit somewhat different characteristics (122, 126).

The sharp decrease in the turbidity of the phospholipid dispersions upon addition of detergent (Fig. 29-31) can be followed photometrically. We have attempted to relate these changes to the rate and extent of hydrolysis of lipid substrates by phospholipase D.

The earlier work of Bangham and Dawson (95-102), which showed that several phospholipases have rather rigorously defined electrokinetic substrate requirements, led to the suggestion that anionic amphipaths might promote hydrolysis by phospholipase D through the provision of an optimal negative surface charge density (18). The present results have therefore to be considered also in this light. (See general discussion in Chapter VI).

The rather rapid decrease of enzymic activity during the reaction period (Fig. 25) and the consequent necessity for high initial concentrations of enzyme, especially in the presence of larger amounts of SDS, may reflect the well known tendency of highly-charged interfaces to denature proteins (156-157).

At low concentrations of added phosphatidic acids, the extent of activation produced by a fixed quantity of the anionic amphipath was independent of whether the disodium salts or free acids were used. However, as the concentration of amphipath was increased, activation by the sodium salts reached a plateau whereas higher concentrations of the free acid proved inhibitory. This is believed to be a pH effect. For negatively charged surfaces the surface pH may be as much as 2 pH units below the bulk pH, mainly because the H^+ ion is behaving as a mobile counterion (158). This explanation was used by Dawson and associates (130) to explain the apparent shift in pH optimum of phospholipase D activated with anionic amphipaths. This effect would be considerably reduced if the Na^+ ion provided by the disodium salt were to partially replace the H^+ ion as counterion.

P. Summary

When diethyl ether was used as activator, 14C, 16C and 18C EPC were poorly hydrolyzed by phospholipase D. When SDS or nonionic detergent (BRIJ 58) was used as activator, the ether analogs were more readily hydrolyzed. Cationic detergent (CTABr) inhibited the hydrolysis of egg-lecithin and the ether analogs. For the hydrolysis of dialkyl ether phospholipids, the optimal molar ratio of lipid:SDS was 1:1. For the hydrolysis of egg-lecithin, the optimal molar ratio of lipid:SDS was 2:1. Anionic detergent (SDS), nonionic detergent (BRIJ 58) and cationic detergent (CTABr) all could disperse the dialkyl ether phospholipids into fine particles.

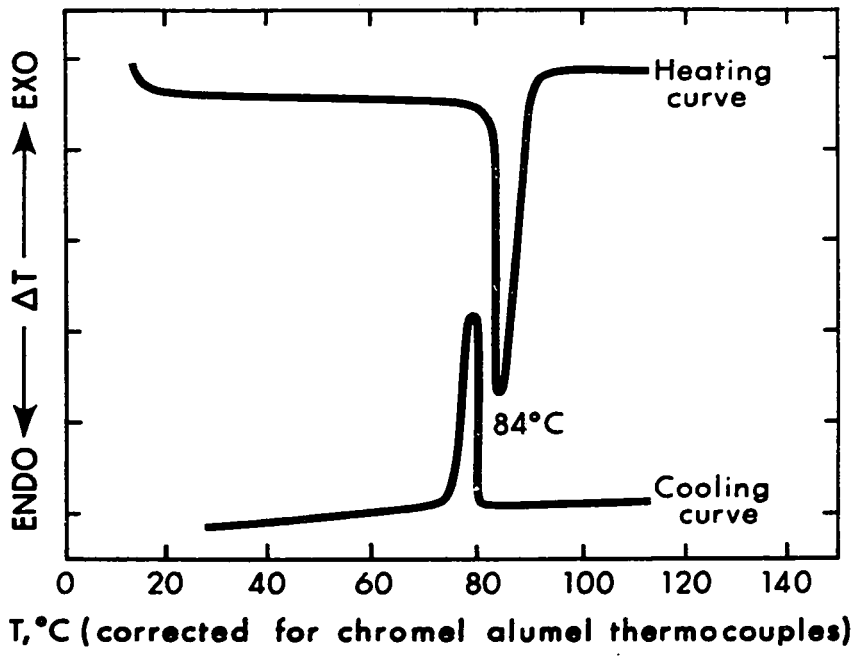
CHAPTER V

THERMAL ANALYSIS OF DIALKYL ETHER PHOSPHOLIPIDS

A. Introduction

"Thermal analysis" originally referred to an experiment in which the temperature of a substance was recorded while it was cooling and was plotted against time to yield a curve showing breaks or inflexions corresponding to thermal transitions in the sample. Currently, two main modifications of this basic technique are in use, differential thermal analysis (DTA) and differential scanning calorimetry (DSC). In DTA, the sample and an inert reference material (glass powder) are heated or cooled at the same rate and the difference in temperature between the sample and reference material is recorded. The differential temperature remains zero or constant until a phase change occurs in the sample. The differential temperature increases until the transition is complete and then decreases again. Thus, a peak is obtained on the curve for differential temperature (ΔT) against temperature or time and the direction of the peak indicates whether the transition is endothermic or exothermic. Fig. 39 shows the transition temperature of 18C EPC in 9% (w/w) of water. In DSC, the temperature of sample and reference are maintained at an equal level or fixed differential throughout the analysis and the variation in heat flow to the sample required to maintain this level during a transition is measured.

Figure 39. D.t.a. curve for 18C EPC in 9% (w/w) water. Sample was prepared as described in Chapter II. In the heating curve, the rate of heating was about 8°C/min.


EXO
ΔT
ENDO
Heating curve
84°C
Cooling curve
0
20
40
60
80
100
120
140
T,°C (corrected for chromel alumel thermocouples)

The DTA and DSC methods have been applied to several biochemical problems. Chapman and his associates (86) have shown that synthetic forms of phosphatidylcholine mixed with 30% (w/w) or more of water, underwent mesomorphic transitions at temperatures much below the transition temperatures for their anhydrous or monohydrated forms. In order to shed some light on the role of cholesterol in biological membranes, Chapman's group studied the thermal transitions of cholesterol-lecithin-water systems (159). They also investigated the thermal behaviour of mixtures of lecithins having different hydrocarbon chain lengths (160). Steim and coworkers (161) have used DSC to investigate the effect of fatty acid composition change on the thermal transition of the biomembrane of Mycoplasma laidlawii. Further thermal studies of Ladbrooke et al. (162) gave information on the organization of lipids in ox brain myelin and in human erythrocyte ghosts.

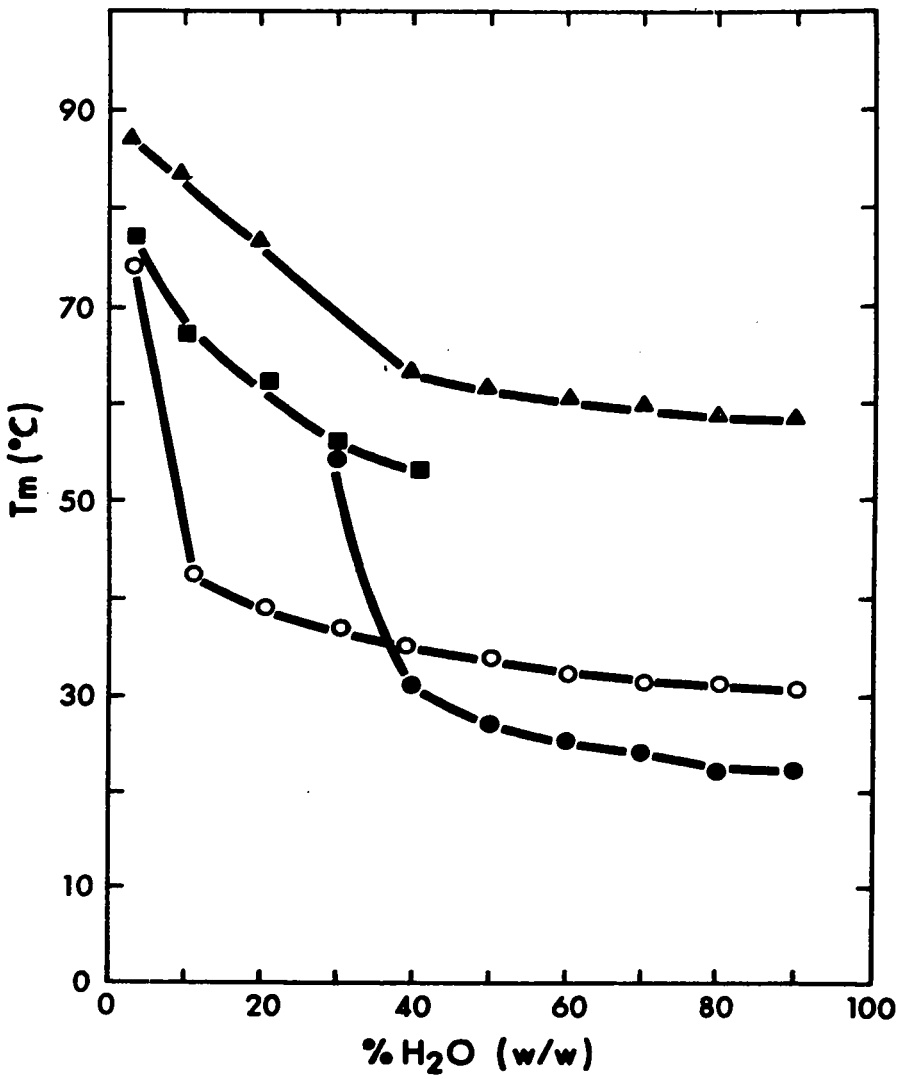
B. DTA of Dialkyl Ether Phospholipids

The different rates of hydrolysis of 14C, 16C and 18C EPC (Fig. 28, Chapter IV) suggested that susceptibility to hydroysis might be related to some property of the hydrocarbon chains. Consequently, we examined the thermotropic and lyotropic behavior of these three dialkyl ether phospholipids using the technique of differential thermal analysis.

1. DTA of Individual Compounds in Different States of Hydration

DTA samples were prepared as described above. Fig. 40 shows the temperatures (Tm) at which the main endothermic transitions were observed for SDS and for dialkyl ether phospholipids in different states of hydration. The thermogram of SDS without added water showed

Figure 40. Effect of water content on the transition temperature of phospholipids and SDS. o, 14C EPC; ▲, 18C EPC, ●, SDS; ■, 1,2-distearoyl-DL-phosphatidylcholine.


90
70
50
30
10
0
Tm (°C)
0
20
40
60
80
100
% H2O (w/w)

three transitions at 17°C, 90°C and 116°C. As the water content was increased, the triplet disappeared and a single major transition was observed at a temperature which decreased with increasing hydration. Each of the dialkyl ether phospholipids showed a single endotherm and in each case the value of Tm decreased with increasing hydration to a limiting value when the water content exceeded 60%.

2. DTA of Systems Containing More than One Component

Fig. 41 shows the melting behavior of mixtures containing various proportions of 14C EPC with 16C EPC and of 14C EPC with 18C EPC, in excess water. For the system 14C EPC - 16C EPC, only one sharp transition peak was observed in each mixture. However, for the system 14C EPC - 18C EPC, the transitions observed were somewhat broader than those obtained with the 14C EPC - 16C EPC system. In both series, a lowering of Tm was observed as the proportion of the shorter chain lipid was increased.

When the chloroform-mixing stage was omitted from the preparation of the sample and instead a mixture of the dry solids was dispersed in water, then two sharp transitions corresponding to the two separate components were observed.

3. Effect of SDS on the Melting Behavior of Hydrocarbon Chains of Dialkyl Ether Phospholipids

The results shown in Fig. 29 were indicative of interaction of dialkyl ether phospholipids with SDS. Therefore, the effect of SDS on the melting behavior of the hydrocarbon chains of dialkyl ether phospholipids was examined (Fig. 42). It can be seen that SDS lowered the transition temperature of each dialkyl ether phospholipid.

Figure 41. **Differential thermal analysis of 14C EPC - 16C EPC and 14C EPC - 18C EPC system in excess water. Composition 25% lipid, 75% water w/w. The temperatures measured from the heating curves.** ●, 14C EPC - 16C EPC; ▲, 14C EPC - 18C EPC.

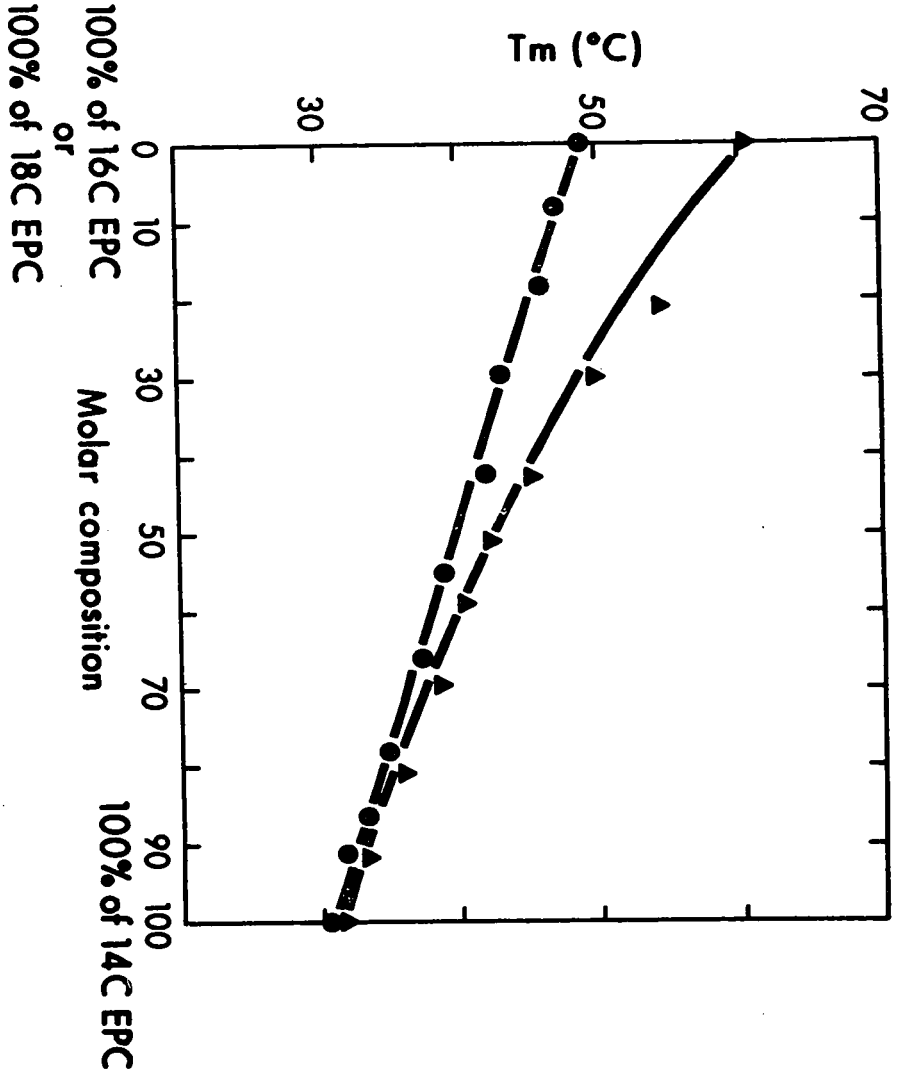
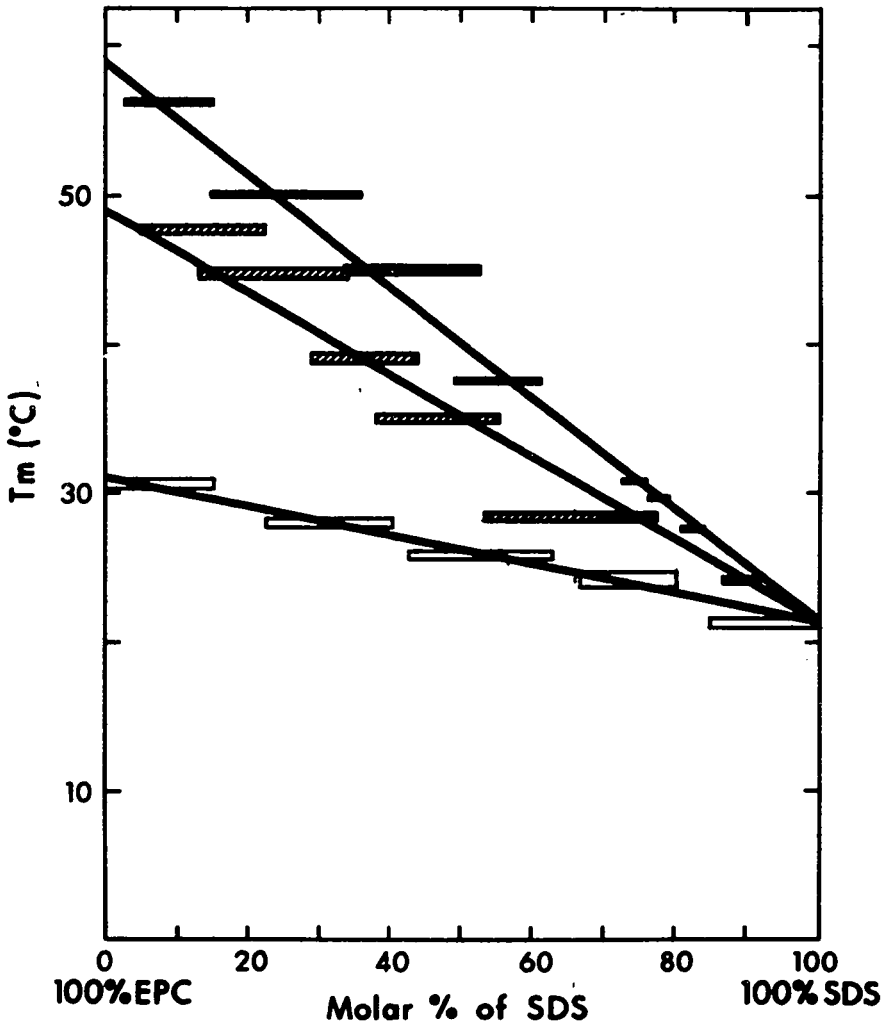

Tm (°C)
30
50
70
0
10
30
50
70
90
100
100% of 16C EPC
or
100% of 18C EPC
Molar composition
100% of 14C EPC

Figure 42. Effect of SDS on the melting behavior of the hydrocarbon chains of dialkyl ether phospholipids in excess water. Composition 25% lipid, 75% water w/w; □, 14C EPC; ▪, 16C EPC; ●, 18C EPC. The width of each bar indicates the range of SDS composition over which a given transition was observed.


50
30
10
Tm (°C)
0
20
40
60
80
100
100%EPC
Molar % of SDS
100%SDS

4. Effect of SDS on the Melting Behavior of Hydrocarbon Chains of Sphingomyelin

Since SDS did not activate the hydrolysis of bovine sphingomyelin, it was of interest to determine the effect of SDS on the melting behavior of the hydrocarbon chains of sphingomyelin. The transition temperature of sphingomyelin in excess water was found to be 42°C. In the presence of SDS, its transition temperature was not affected.

C. Discussion

Phospholipids placed in an aqueous environment will swell spontaneously and form liquid crystals only when the environmental temperature exceeds the melting temperature of the hydrocarbon chains within the gel structure (86). Thus, a gel to liquid crystalline transition can be induced by raising the temperature, by decreasing the chain length, by increasing the extent of unsaturation or by the introduction of substituents or molecules which promote disorder in the hydrocarbon region (159). The requisite temperature for the phase transition (Tm) in any particular case can be conveniently determined by differential thermal analysis (86).

Values of Tm for 1,2-distearoyl lecithin (58°C), 1,2-dipalmitoyl lecithin (42°C) and 1,2-dimyristoyl lecithin (24°C) in excess water have been reported (160). They are slightly lower than those found for the corresponding dialkyl ethers (Tm 60°C, 49°C and 32°C respectively). The differences between the diesters and the diethers, most marked with the shorter chain lengths, are probably due to differences in the lyotropic component of the mesomorphic transition.

A structural difference is also manifested in the higher surface potentials of the diesters (∿ 400 mV) as compared with the diethers (∿ 200 mV) when measured electrometrically in a monolayer (163).

It has been possible to show that the extent of hydrolysis of three homologous dialkyl ether phospholipids containing 14C, 16C and 18C substituents is inversely related to their Tm values (32°C, 48°C and 60°C respectively) in excess water (Table II in Chapter IV and Fig. 40 in Chapter V). None of these compounds was hydrolyzed as rapidly as egg lecithin (Tm < 0°C) under comparable conditions. The addition of a short chain amphipath (SDS or 14C EPA) or a partially unsaturated lipid (egg PA or egg Na_2PA) tended to decrease Tm and promote hydrolysis (Figs. 27, 29, 33, and 35 in Chapter IV). The addition instead of comparable longer chain saturated phospholipids (16C EPA or 18C EPA) did not have this effect (Fig. 35). Furthermore, the saturated phospholipids when mixed with egg lecithin decreased the extent of its hydrolysis. This inhibitory effect, observed only in the presence of SDS, appeared to be competitive with respect to the available SDS (Fig. 36 in Chapter IV).

Temperature-composition diagrams for binary mixtures of saturated lecithins in excess water have also been reported (160). Ideal mixing and co-crystallization were observed when the difference in chain lengths was 2C atoms but not when the difference was 4 C atoms. In the present study (Fig. 41), ideal mixing was observed for the system 14C EPC-16C EPC but the system 14C EPC-18C EPC appeared to be marginal in this respect. The preparation of the binary sample was shown to be quite critical in this context.

During the course of this investigation, Abramson (164) reported on the lyotropic behavior of L-2,3-dihexadecyl-glycerine-1-phosphorylcholine and L-2,3-dihexadecyl-glycerine-1-phosphoryl-ethanolamine. These compounds, containing the ether linkage to glycerol in place of the ester linkage, also exhibited transitions at slightly higher temperatures than their diacyl analogs.

D. Summary

The thermotropic and lyotropic behaviour of 14C, 16C, and 18C EPC was examined by using the technique of differential thermal analysis. Each of the dialkyl ether phospholipids showed a single endotherm and in each case the value of Tm decreased with increasing hydration to a limiting value when the water content exceeded 60%. Ideal mixing was observed for the system 14C EPC - 16C EPC but the system 14C EPC - 18C EPC appeared to be marginal in this respect. SDS can lower the transition temperature of each dialkyl ether phospholipid but does not lower the transition temperature of bovine sphingomyelin.

CHAPTER VI

GENERAL DISCUSSION

This thesis is principally concerned with those aspects of the phospholipase D enzymic reaction which reflect the physicochemical nature of the substrate. Bangham and Dawson (95-102) have attempted to show that various phospholipases will hydrolyze lecithins only when activated with anionic or cationic detergents. The activators were shown to introduce on the surface of the substrate particles, a net surplus of charges which were thought necessary for the initiation of the enzyme attack. However, from studies of the mechanism of diethyl ether activation of lecithin hydrolysis by snake venom phospholipase A_2, Dawson (101-102) concluded that penetration of the diethyl ether molecules into the lipid particles causes a wider spacing of the substrate molecules. Kates (128) interpreted the role of diethyl ether penetration as increasing the lipophilic nature of the substrate. These factors were assumed to improve the access of the enzyme to the reaction site on the lipid substrate molecules. Although the importance of liquid-crystallinity in membranes has been heavily stressed recently (87), the present work is the first instance in which it has been clearly demonstrated that a liquid-crystalline substrate is required for the activity of a lipolytic enzyme.

From the experimental results described in Chapter IV and Chapter V, it is suggested that the rate of hydrolysis of the phosphate diester bond in the polar head groups of the phospholipid molecules by phospholipase D is determined by: (1) the surface area available to

the enzyme; (2) the separation of the polar head groups in the lipid-water interface; (3) the fluidity of the hydrocarbon chains of the phospholipid molecules; (4) the surface charge density.

Surface Area

If the lipid substrate is present as a large multilamellar structure, the external surface area available to the enzyme must be quite limited. However, if the lipid substrate is ultrasonically irradiated and forms small vesicles, each bounded by a single bilayer, the surface area available for enzyme attack will increase to a maximum of 50% of the total surface area. If the lipid molecules are in the form of monolayers or spherical micelles, the surface area available to the enzyme will increase to 100%. This argument assumes that the protein cannot pass completely through the lipid layer and that the lamellar structures are formed into "closed" compartments.

Phospholipids when placed in an aqueous environment will swell spontaneously and form liquid crystals only when the temperature exceeds the melting temperature of the hydrocarbon chains within the gel structure (86). Swelling (hydration) is indicative of an interaction of the lipid molecules with the aqueous solvent. This should permit greater accessibility of the substrate to the enzyme. The decrease in optical density of the phospholipids in the presence of SDS, BRIJ 58 or CTABr is certainly indicative of a reduction in the particle size and, concomitantly, an increase in the area of available lipid-water interface. Monochain compounds, such as SDS, BRIJ 58 and CTABr, tend to promote the formation of small mixed micelles with maximal surface area available to enzyme while egg PA and synthetic EPA tend to

favor extensive multilamellar systems with limited external surface area. This may help to explain the greater effectiveness of SDS or BRIJ 58 in promoting enzymic hydrolysis. Although egg lecithin is already in a liquid-crystalline state at room temperature in the presence of excess water, it is not hydrolyzed by phospholipase A or phospholipase D unless activators, such as SDS, are added. This is probably due in part to the fact that egg lecithin disperses to form extensive multilamellar structures.

Separation of the Polar Head Groups in the Lipid-Water Interface

Studies of X-ray diffraction spacings (84) indicated that for soaps the sequence gel - hexagonal liquid crystal - complex hexagonal - cubic - lamellar is accompanied by a monotonic increase in the area(S) occupied by each head group. It has also been demonstrated that phospholipid bilayers thin out during a gel-liquid crystal transition, an effect accompanied by increases in the polar head group spacings (84). It seems reasonable to assume that a spacing out effect accompanies the major gel-liquid crystalline transitions reported in this thesis. It is suggested that the head groups of the phospholipid molecules may have to be literally pushed apart in order for the enzyme to gain access. This effect would also be achieved by interpolation of detergent molecules, diethyl ether or water molecules between adjacent phospholipid molecules.

Fluidity of Hydrocarbon Chains of the Phospholipid Molecules

Fluidity of the hydrocarbon chains in the internal structure of the lipid phase may promote better binding of the enzymic proteins to lipid substrates. It has been shown that there was negligible binding

of enzyme to 14C EPC under conditions in which considerable binding to egg lecithin occurred (Table V, Chapter IV). Recently, Juliano, Kimelberg and Papahadjopoulos (165) have reported on the synergistic effects of a membrane protein (Spectrin) and Ca^{+2} on the Na^{+} permeability of phospholipid vesicles. They suggested that an initial electrostatic attraction followed by hydrophobic interaction between protein and lipid mediated a partial penetration of the protein into the phospholipid bilayer, resulting in the large increase in Na^{+} permeability. Such a hydrophobic interaction would be greatly facilitated if the hydrocarbon chains of the phospholipid molecules were in a liquid-crystalline state.

Surface Charge Density

This has been discussed above.

Some of the experimental results reported in the preceding chapters may now be interpreted in the light of these general features.

1. Activation by Diethyl Ether

The results shown in Table III (Chapter IV) indicated that in the presence of diethyl ether the enzymic hydrolysis took place at the lipid-water interface. It is conceivable that under these conditions, an oriented monolayer of the substrate molecules was formed at the solvent-water interface with a free exchange of the lipid molecules between this interface and the bulk aqueous phase. The hydrocarbon chains of the lipid molecules would be directed into the diethyl ether phase (saturated with water) and the polar head groups into the water phase (saturated with diethyl ether). The free motion of the hydrocarbon chains in the ethereal phase would cause a wider spacing of

the polar head groups of the phospholipid molecules, allowing easier penetration of enzymic protein into the lipid monolayer. The lower solubility of dialkyl ether phospholipids in diethyl ether as compared with the corresponding diacyl compounds would result in a lowered tendency to form an interfacial monolayer, thereby accounting for the much lower susceptibility to hydrolysis of the former group of compounds and also for their lack of effect on the hydrolysis of egg lecithin.

Heller and Arad (122) have indicated that the reaction of soluble phospholipase D from peanuts with egg lecithin takes place in the aqueous phase of the diethyl ether-water system. In their experiments, they used a rather low enzyme concentration (0.5 mg/ml). In Chapter IV, it is shown that, with a higher concentration (4 mg/ml) of soluble phospholipase D from cabbage leaf, the hydrolysis of egg lecithin occurs at the diethyl ether-water interface. In order to check the results of Heller and Arad (122), a lower concentration (0.5 mg/ml) of cabbage leaf phospholipase D was then used. It was found that a very thin interface was formed between the diethyl ether and water phases (Chapter IV-B). Inclusion of this very thin interface with the aqueous phase, as in the work of Heller and Arad (122), gave the false impression that the reaction took place in the bulk aqueous phase.

As noted above, Kates (128) suggested that diethyl ether makes the surfaces of the phospholipid substrate and of the plastid enzyme sufficiently lipophilic for coalescence and hydrolysis to occur. This appears to be applicable to the case of the soluble enzyme only if

it is assumed that apolar groups of the enzyme are involved in the lipid-protein interaction.

2. Activation by Anionic Amphipathic Compounds

As regards the activation effect of anionic amphipaths, the interpolation of negatively charged groups, resulting in mutual electrostatic repulsions, would also be expected to cause spacing out of the phospholipid polar head groups. Anionic detergents, such as SDS, would also be expected to increase the surface area of the substrate available for enzyme attack by forming small mixed micelles.

3. Activation by Nonionic Detergent (BRIJ 58)

A nonionic detergent (BRIJ 58) has been found to be an activator for the enzymic hydrolysis (Tables VI and VII, Chapter IV). The net surface charge density in this case would approach zero. This activation may be due to an increase in the available surface area for enzyme attack or to spacing out the polar head groups of the phospholipid molecules. It should be noted,however,that activation by BRIJ 58 was only 80% that of the SDS control experiment. Consequently, an additional activation by SDS due to the provision of a negative surface charge density cannot be ruled out.

The effect of BRIJ 58 on the melting behavior of the hydrocarbon chains of dialkyl ether phospholipids was not examined because of a lack of sufficient quantities of the substrates.

4. Effect of Cationic Detergent (CTABr)

Although CTABr had the same type of effect as SDS or BRIJ 58 in dispersing dialkyl ether phospholipids to an isotropic solution (Fig. 30, Chapter IV), it was a potent inhibitor of phospholipase D action (Reference 106 and Figure 32, Chapter IV). However, when excess SDS was initially present (14C EPC:SDS = 1:1.5 molar ratio), such that the system was inhibited by the excess of anionic detergent, then addition of CTABr reversed this inhibition (Figure 32, Chapter IV). This result would also support the idea that an appropriate surface charge density determines to some extent the rate of enzymic hydrolysis.

5. Activation by Ultrasonic Irradiation of the Substrate

Dawson and his associates (104, 130) have shown that ultrasonic dispersionsof egg lecithin can be readily hydrolyzed by phospholipase A_2 of snake venom (Naja naja) or by the soluble phospholipase D of cabbage leaf. Kates (105) also demonstrated that the ultrasonically dispersed egg lecithin can be readily hydrolyzed by Penicillium notatum phospholipase B in the absence of any activator. Many workers have assumed that ultrasonication of phospholipid merely breaks down the extensive multilamellar structure to smaller particles or vesicles having the same basic molecular arrangement (166-169). However, in contrast with coarse dispersions, ultrasonically irradiated preparations of lecithin in D_2O give high-resolution nuclear magnetic resonance spectra (107) in which the peak areas reflect the proportion of the phospholipid protons which contribute to the spectrum. This result cannot be easily interpreted as being due merely to a reduction in particle size (108). It implies that sonication causes

a rearrangement of the structure at the molecular level. Such structural changes have interesting consequences. For example, a phospholipase A derived from lysosomes of the bovine adrenal medulla (170) is unable to hydrolyze lecithin which has simply been swollen in aqueous solution. After ultrasonic irradiation hydrolysis can be observed; this cannot be accounted for on the basis of the increase in substrate surface area. Sheard (108) has suggested that a loosening of the structure might allow enzyme access to the ester linkage of the hydrocarbon chains in the phospholipid molecules.

6. Effect of Diester and Diether Linkagesof the Hydrocarbon Chains in the Phospholipid Molecules

Values of T_m for 1,2-distearoyl lecithin (58°C), 1,2-dipalmitoyl lecithin (42°C) and 1,2-dimyristoyl lecithin (24°C) in excess water have been reported (160). They are slightly lower than those found for the corresponding dialkyl ethers. (T_m 60°C, 49°C, and 32°C, respectively) (Figure 40, Chapter V). The differences between the diesters and diethers, most marked with the shorter chain lengths, are probably due to differences in the lyotropic components of the mesomorphic transitions. Abramson (164) also observed that the dialkyl ether phospholipids exhibited transitions at slightly higher temperatures than their diacyl analogues in excess water. He suggested that this difference might be the result of closer packing of the hydrocarbon chains with increased van der Waals forces in the ether bonded compounds.

In a preliminary study, the surface potential (ΔV) due to a monolayer of lecithin molecules (+ 400 mv) was found to be much greater than that due to a monolayer of dialkyl ether phospholipid molecules (+ 200 mv) having the same polar head group and hydrocarbon chain type. The surface potential (ΔV) of a monolayer depends primarily on the characteristics of the polar groups in the monolayers. If a monolayer is spread or adsorbed on a clean water surface, the water dipoles will generally be re-orientated about the film-forming molecules because of the new dipoles introduced into the surface. This change will be denoted by μ_1. The dipoles of the film-forming molecules themselves will also contribute to ΔV by an amount depending on the polar group dipole moments, μ_2. Any other contributions of dipole moments to ΔV can be denoted by μ_3, μ_4, etc. If we apply the Helmholtz formula for an array of n dipoles per cm^2, and if they are all vectorially additive in the vertical direction, we obtain: (171)

$$\Delta V = 4\pi n\mu_1 + 4\pi n\mu_2 + 4\pi n\mu_3 + \text{----------}$$

The observed difference in ΔV between diester and diether linkages of the hydrocarbon chains in the phospholipid molecules could then possibly indicate a much greater dipole moment (or induced series of dipole moments) at the level of $-O-\overset{O}{\overset{\|}{C}}-$ as compared with $-O-CH_2-$. Consequently, the attraction for polar molecules of solvent to this region could be lowered for the diether type linkage. In practice, this appears to result in an enhanced swelling of diacyl compounds in water as compared with the diethers. This could explain the observation that, when all other known variables are kept constant, the diesters are more rapidly hydrolyzed than the analogous diethers.

7. Effect of Cholesterol on the Hydrolysis of Sonicated Egg Lecithin

Since two or more modifications in the physical structure of the substrate may occur simultaneously, it is often difficult to relate changes in enzymic hydrolysis directly to one particular effect. As an illustration of this point, the following experiments may be cited:

Egg lecithin (8 μmoles) was dissolved in $CHCl_3$ and various amounts of cholesterol (0 -10 μmoles) in $CHCl_3$ were added to the egg lecithin solution. The solvent was removed with a stream of nitrogen and then dried *in vacuo* overnight. 0.625 ml of 0.1 M acetate buffer, pH 5.0 was added and the lipid mixture exposed to ultrasonication for 30 minutes. After 0.1 ml of 1 M $CaCl_2$ solution and 0.5 ml of phospholipase D (4 mg, type I) had been added, the mixture was incubated at 26°C for 2 hours. It was found that 65% of the sonicated egg lecithin (without cholesterol) was hydrolyzed in the two hour period and that inclusion of cholesterol (1-10 μmoles) did not alter this result.

The effects of cholesterol on egg lecithin have been examined in some detail (107, 172). In mixed monolayers at a given surface pressure, the area occupied by the cholesterol and phospholipid molecules is less than would be expected from the sum of the areas occupied by these molecules present in separate monolayers at the same surface pressure. Cholesterol is therefore considered to have a condensing effect on egg lecithin monolayers. Since cholesterol is a rather rigid molecule, this condensation most likely originates from a reduction in the volume excluded by the lecithin molecules. In agreement with this idea, NMR studies suggest that cholesterol inhibits the

molecular motion of the hydrocarbon chains of the phospholipid in the liquid crystalline state (107). Nonetheless, interpolation of cholesterol molecules into monolayers or bilayers of lecithin is expected to increase the net separation of the phospholipid polar head groups to some extent. From X-ray diffraction data of phospholipid-cholesterol-water systems (172), it can be calculated that the linear separation of the polar head groups increases by about 20% after addition of 50 mole % cholesterol.

The failure of cholesterol to influence the rate of enzymic hydrolysis of sonicated egg lecithin could be interpreted as follows: Provided that substrate (egg lecithin alone) were in a liquid crystalline condition, the enzymic protein could penetrate between the polar head groups, permitting a subsequent hydrophobic interaction of the protein with the lipid chains. Following the work of Juliano, Kimelberg and Papahadjopoulos (165) such an interaction would have the effect of distorting the lipid bilayer causing further small separation of the polar head groups to allow maximal enzyme-substrate binding. Addition of cholesterol to the substrate would itself produce greater separation of the polar head groups but would concomitantly reduce the possibility of protein penetration into the lipid bilayer. In the presence of enzyme therefore, the net separation of the polar head groups could be the same regardless of whether cholesterol were present or not.

In summary, it is suggested that the rate of hydrolysis of phospholipid molecules by phospholipase D is controlled by four factors.

i. the interfacial area which is available for enzyme attack.

ii. the ability of the enzyme to penetrate between the polar head groups prior to attack on the phosphatediester bond.

iii. a postulated secondary interaction dependent on the fluid condition of the hydrocarbon chains and involving hydrophobic interaction between lipid and enzyme.

iv. the surface charge density controlling the initial approach of the enzyme to the substrate.

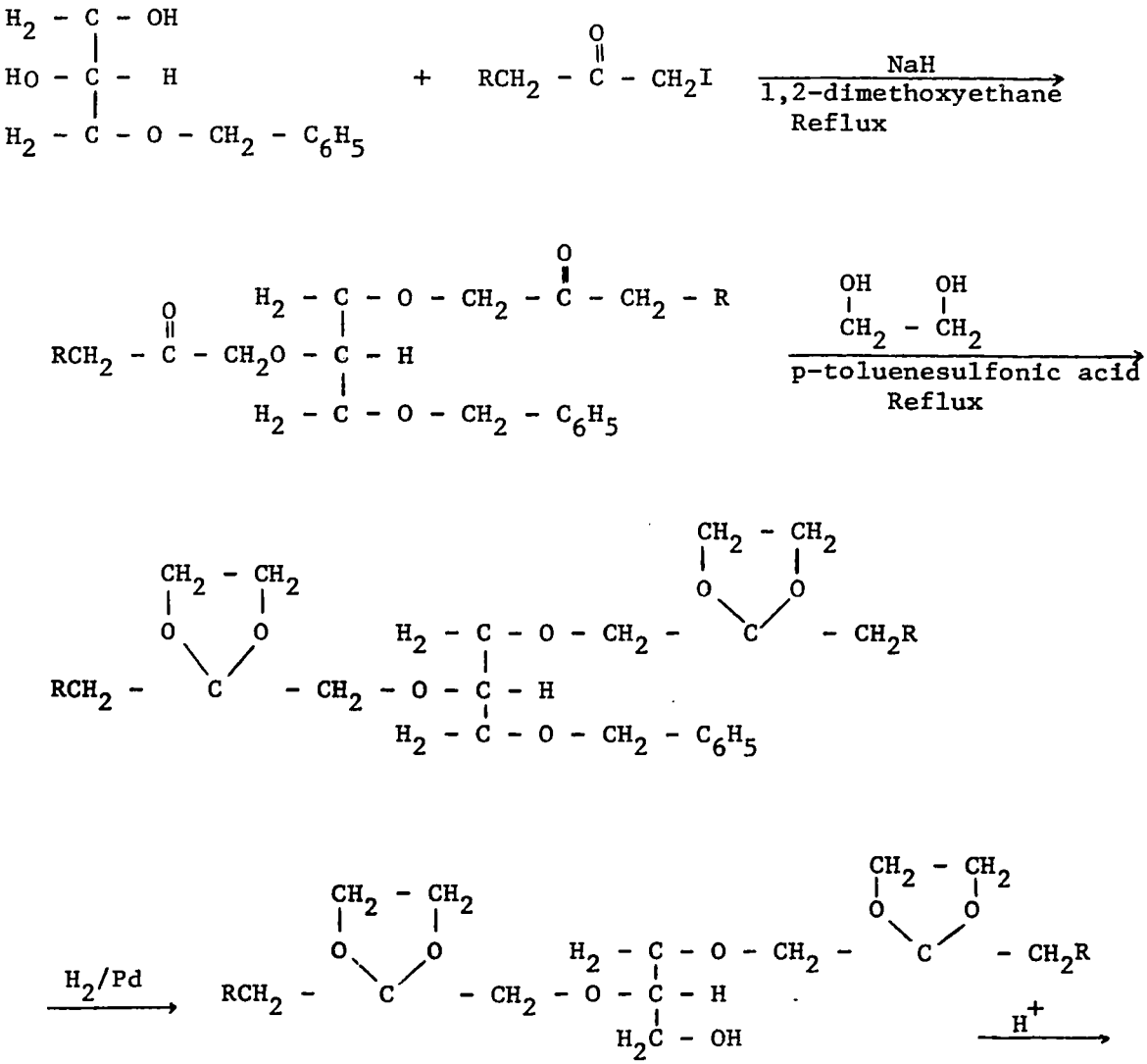
Proposal for Future Work

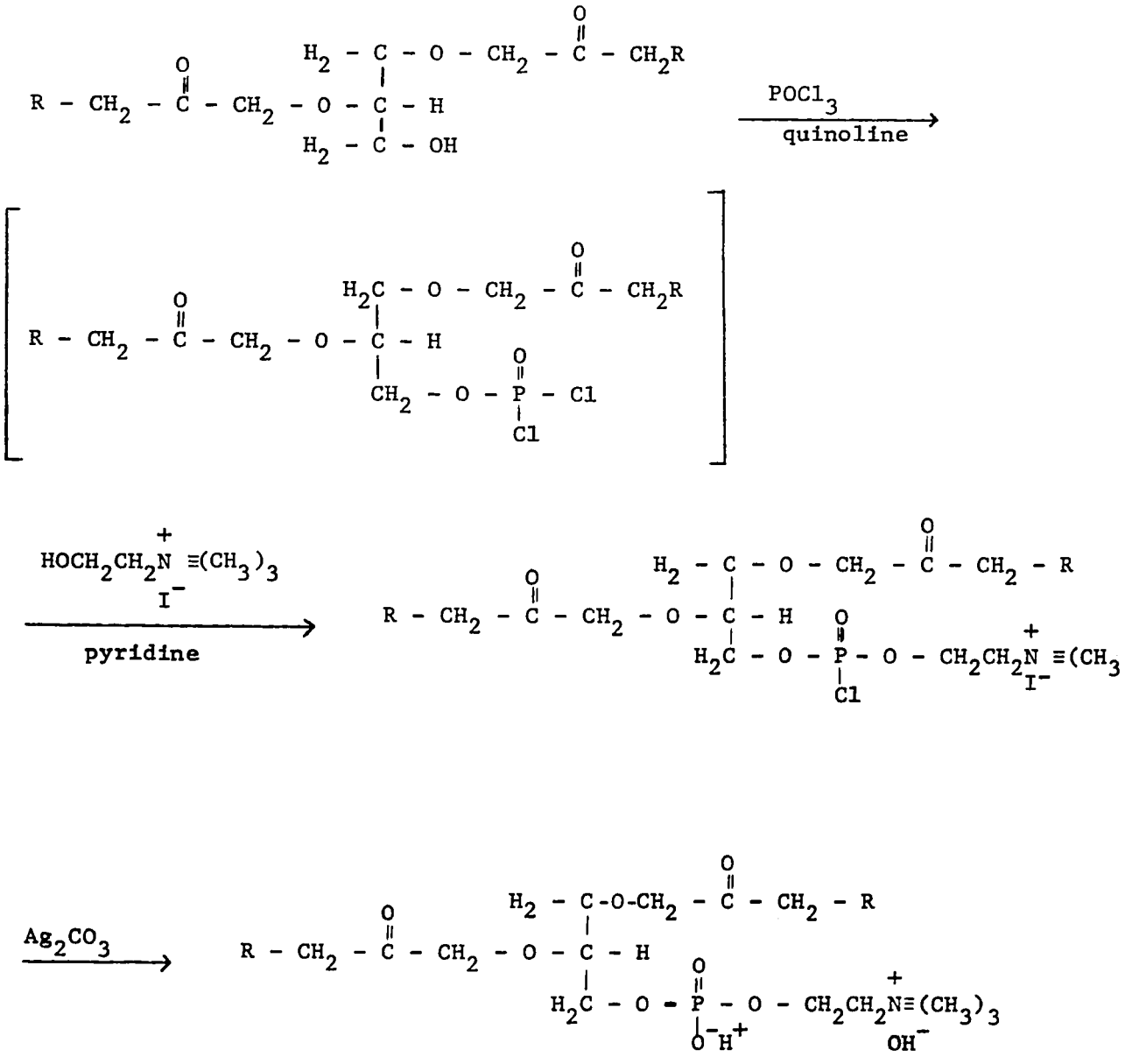
1. Chemical Synthesis of α-Keto Ether Analogs. Examination of their Physical Properties and Susceptibilities to Enzymic Attack

Further modification of the linkages between the hydrocarbon residues and the glyceryl backbone followed by examination of the lyotropic and thermotropic mesomorphism and susceptibility to hydrolysis would allow more definitive statements to be made about the parameters discussed above. Such studies would also be useful in understanding the properties of naturally occurring diacyl lipids as they exist in a a biological environment. For example, the α-keto ether derivatives might usefully serve this purpose.

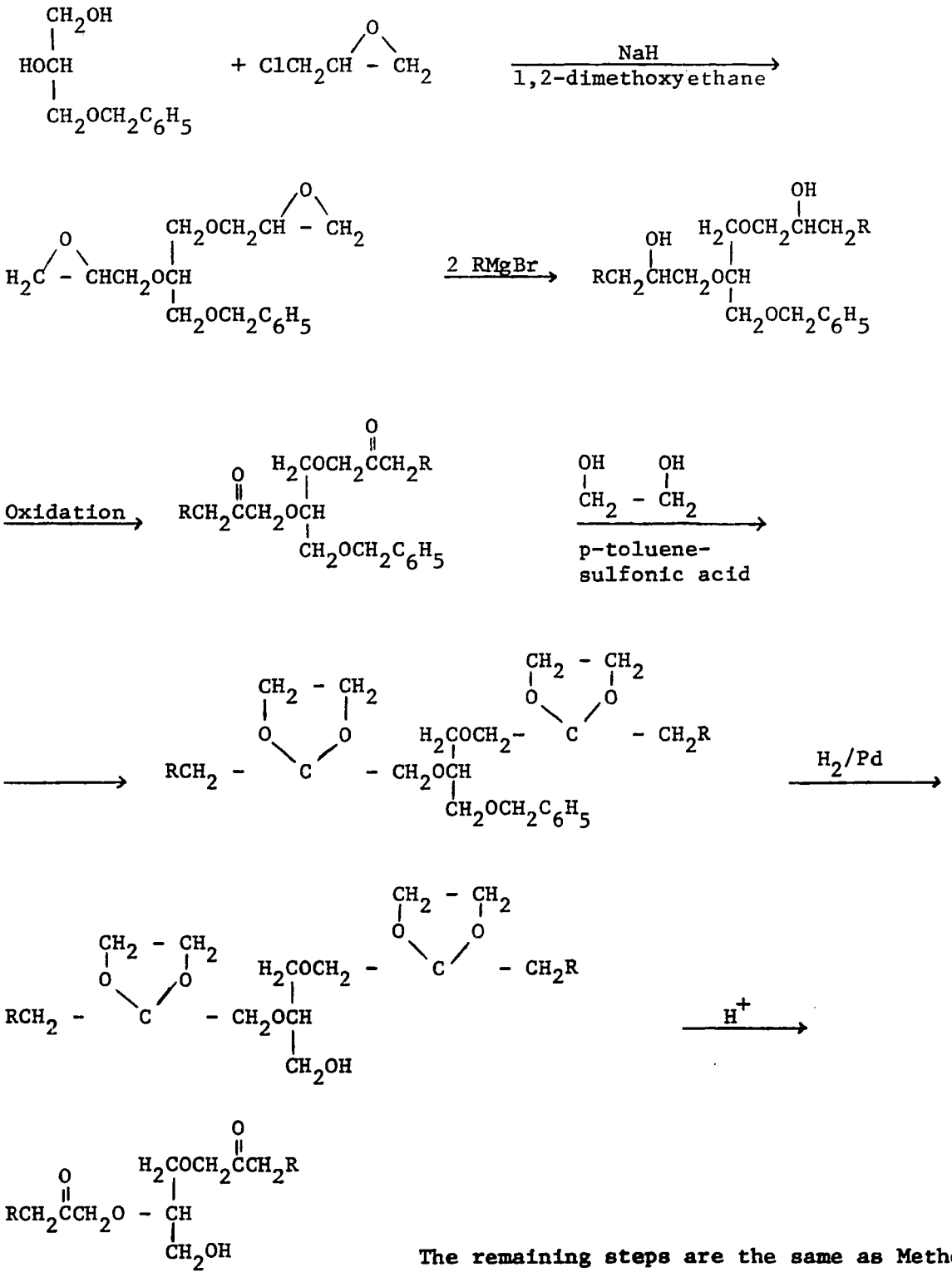
The following reaction schemes are suggested for the synthesis of α-keto ether phospholipids.

Method A





Method B



The remaining steps are the same as Method A.

2. Purification of Phospholipase D from Cabbage Leaf

The stability of the soluble phospholipase D is highly dependent on its state of purity. In crude preparations, the activity remains stable for 5 minutes at 55°C (112). This activity becomes progressively more thermolabile on purification (112). In an unpublished experiment, it was found that EPC would inhibit the hydrolysis of egg lecithin when stage I enzyme preparation (112) and standard reaction mixture A were used. However, when stage III enzyme preparation was used, EPC did not inhibit the hydrolysis of egg lecithin (Figure 22, Chapter IV). Hence, it would be of considerable interest to examine more highly purified forms of phospholipase D and to study the hydrolysis of phospholipid molecules with this enzyme.

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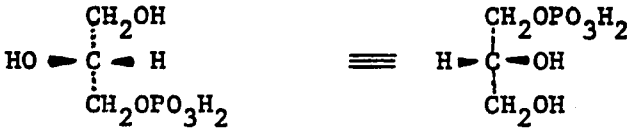
APPENDIX I

The Nomenclature of Lipids (173)

Although the systematic names of individual lipids can be derived by the general rules of organic nomenclature, such names are often complex. The following systems have conventionally been used in the lipid field.

A. Baer - Fischer System

According to this system "an α-monoglyceride is put in the same category with that glyceraldehyde into which it could be transformed by oxidation without any alteration or removal of substituents". This system has been criticized by Brown, Clark and Letters (174) who stated that "confusion can, and does, arise from whether α refers to the 1 or the 3 position" and by Baddiley, Buchanan and Carss(175) who pointed out that the correct name for the naturally occurring L-α-glycerophosphate (I) according to standard rule of nomenclature is D-glycerol 1-phosphate (II).



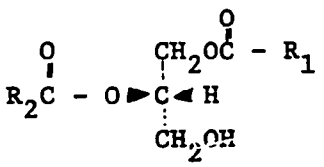
I

II

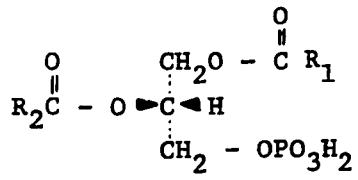
B. A second conventional nomenclature also employs D/L prefixes, uses numerals as locators and usually gives the substituted primary carbinol group the lower number (176). The coexistence of these two systems which usually employ antipodal configurational prefixes for the same substance has been a source of some confusion and ambiguity.

C. R/S System

This system has been recommended as the general system because of its universal character and its freedom from ambiguity. However, large numbers of chemical and biochemical reactions involve the formation and cleavage of ester and ether linkages to glycerol. The description of these processes under the rules of R/S or D/L system requires frequent changes of the configurational prefixes. For example, the phosphorylation of (S)-1,2-diacylglycerol (III) gives an (R)-phosphatidic acid (IV).



III

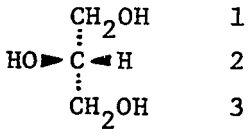


IV

D. Stereospecific Numbering System

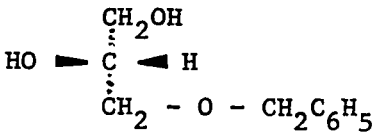
In 1963, the Biochemical Nomenclature Commission of IUPAC and the Commission of Editors of Biochemical Journals of IUB set up an International Sub-Committee on Lipid Nomenclature. A new nomenclature, the stereospecific numbering system, which could readily be applied to glycerol derivatives was then proposed.

If the secondary hydroxy group is shown to the left of C-2 in a Fischer projection, the carbon atom above C-2 is called C-1, the one below C-3.



The use of this stereospecific numbering system is indicated by the prefix "sn" before the stem-name of the compound. It is also used to describe the stereochemistry of glycerol derivatives by indicating the carbon atoms that are substituted. This system has now been widely adopted in the lipid field.

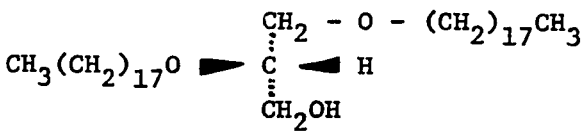
Example 1.



Baer-Fischer system: L-α-benzylglycerol.

sn system: 3-benzyl-sn-glycerol.

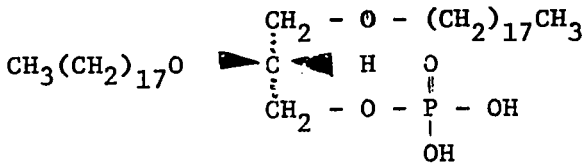
Example 2.



Baer-Fischer system: D-α,β-di-O-octadecylglycerol

sn system: 1,2-dioctadecyl-sn-glycerol.

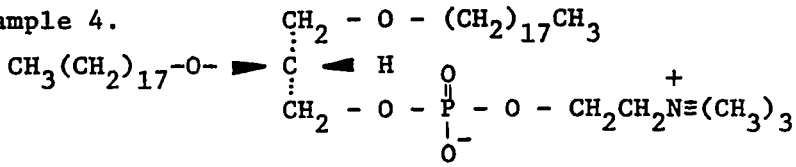
Example 3.



Baer-Fischer system: Di-O-octadecyl-L-α-glyceryl-phosphoric acid.

sn system: 1,2-dioctadecyl-sn-glycero-3-phosphoric acid.

Example 4.



Baer-Fischer system: Di-O-octadecyl-L-α-glyceryl-phosphorylcholine

sn system: 1,2-dioctadecyl-sn-glycero-3-phosphorylcholine.

APPENDIX II

Chemical Synthesis of Mixed 1,2-Dialkyl-sn-Glycerol

The following intermediates were synthesized during the course of this work but the desired final product was not obtained. The partial syntheses are therefore reported as a separate appendix.

Chacko and Hanahan (28) described the partial synthesis of 1,2-alkylalkenyl-sn-glycerol from selachyl alcohol (1-cis-9'-octadecenyl-sn-glycerol) which was isolated from dog fish liver oil. In the following experiments, it was intended to synthesize mixed 1,2-dialkyl-sn-glycerol from 1,2-isopropylidene-sn-glycerol. This new approach can be applied to the synthesis of 1,2-dialkyl-sn-glycerol with different hydrocarbon chains on C_1 and C_2 positions.

A. Preparation of 1-O-triphenylmethyl-3-benzyl-sn-glycerol (D-α-O-triphenylmethyl-α'-benzyl glycerol)

A solution of 16.46 g (0.09 mole) of L-α-benzyl glycerol and 50 g (0.18 mole) of triphenylchloromethane in 170 ml of anhydrous pyridine was stirred at room temperature for 22 hours. The reaction mixture was added to ice-cold water (350 ml) and extracted with diethyl ether (4 x 400 ml). The diethyl ether solution was evaporated to 450 ml under reduced pressure and then washed successively with 2 x 250 ml 1N H_2SO_4, 2 x 250 ml water, 2 x 250 ml of 5% $NaHCO_3$ and 2 x 250 ml water. The ether solution was then dried over anhydrous sodium sulfate and the solvent removed under reduced pressure. The dried residual oil was dissolved in 200 ml of a mixture of hexane and benzene (1:1 v/v). The insoluble material was removed, the

supernatant was evaporated under reduced pressure and was dissolved in 60 ml of a mixture of hexane and benzene (2:1 v/v). The insoluble material was again removed. The supernatant was evaporated to dryness and redissolved in 60 ml of a mixture of hexane and benzene (2:1 v/v). The insoluble material was again removed. This process removed most of the triphenylcarbinol. The supernatant, which contained most of the desired product, was collected and the solvent removed under reduced pressure. The residual yellow oil, weighing 38 g, was dissolved in 60 ml of a mixture of hexane and benzene (2:1 v/v) and passed through a column of silicic acid (5 cm diameter, 400 g of Silic ARCC 7, 200 g celite) in hexane. The column was washed with 10 liters of hexane-benzene mixture (2:1 v/v), 1.5 liters of hexane-benzene (1:1 v/v) and 800 ml of hexane-benzene (1:2 v/v) to remove a yellow coloured component visible after TLC. Then, 5 liters of chloroform were used to wash off the desired product. The chloroform fractions were combined, the solvent was removed under reduced pressure and the residual oil was dried <u>in vacuo</u> overnight. The yield was 33.7 g (89% of theory). $[\alpha]_D^{25}$ = -0.57 (C 8.7% w/v, in chloroform).

Thin-layer chromatography on Silica gel G with solvent A gave an R_F of 0.13.

Infrared absorption spectrum (see Figure 43).

Nuclear magnetic resonance spectrum (see Figure 44).

Anal. Calcd. for $C_{29}H_{28}O_3$ (424.54): C, 82.05; H, 6.65. Found: C, 82.41; H, 6.53.

Figure 43. Infrared absorption spectrum of 1-O-triphenylmethyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophotometer.

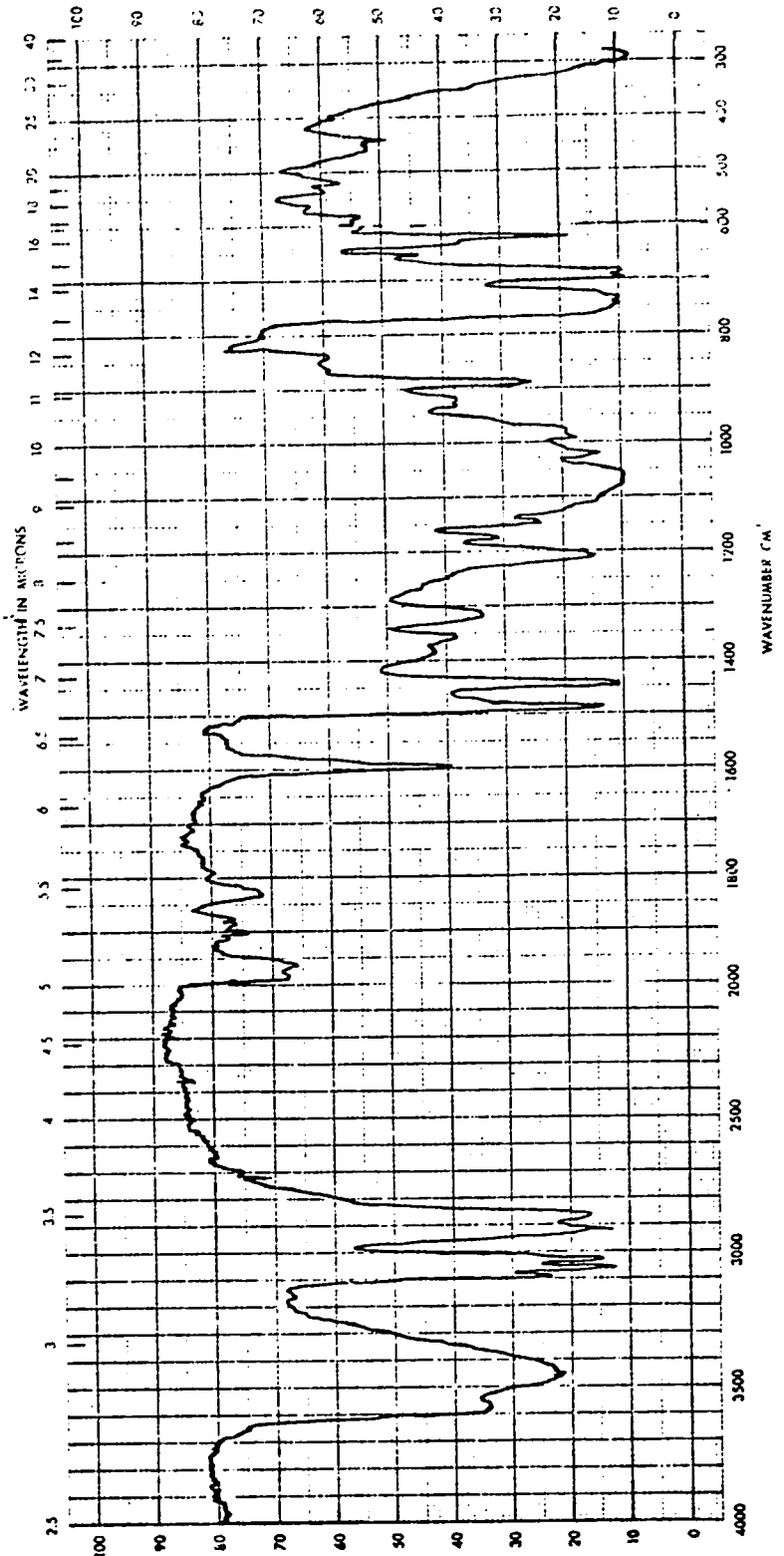
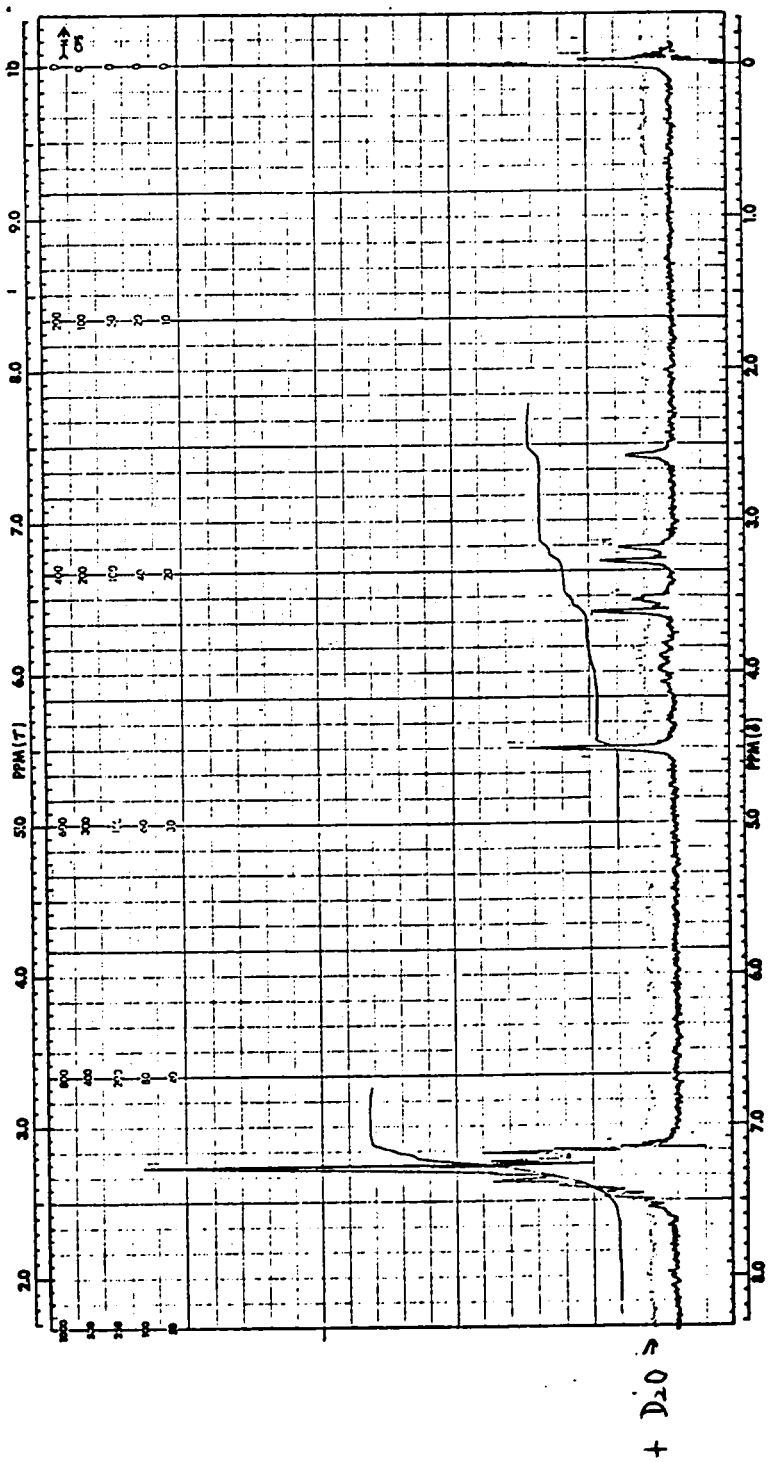

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 44. Nuclear magnetic resonance spectrum of 1-O-triphenylmethyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


δ
PPM(τ)
PPM(δ)
+ D2O

B. Preparation of 1-O-triphenylmethyl-2-octadecyl-3-benzyl-sn-glycerol (D-α-O-triphenylmethyl-β-O-octadecyl-α'-benzyl glycerol)

13.25 g (33.6 mmole) of 1-O-triphenylmethyl-3-benzyl-sn-glycerol was dissolved in 400 ml of dry benzene, 15 g powdered KOH was added and the mixture was refluxed with stirring, the water formed being removed into a separating head (about 2 hours). Then, 17.43 g (50 mmole) of n-octadecylmethane sulfonate in 140 ml of dry benzene was added through a dropping funnel. The mixture was refluxed with stirring for 20 hours. After the reaction mixture had cooled to room temperature, 600 ml of water was added and the mixture extracted with 3 x 600 ml diethyl ether. The organic phases were combined and the volume was reduced to 500 ml under reduced pressure. The ether solution was then washed successively with 2 x 250 ml 0.5 N H_2SO_4, 2 x 250 ml H_2O, 2 x 250 ml 5% $NaHCO_3$ and 2 x 250 ml water. The organic layer was then dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The residual oil was dissolved in a minimum volume of hexane and chromatographed on a column (5 cm diameter, 400 g Silic ARCC 7, 200 g celite) in hexane. The column was washed with 10 liters of 0.7% diethyl ether in hexane, then the desired product was washed off with 6 liters of 1.5% diethyl ether in hexane. The fractions of the desired compound were combined and the solvent was removed under reduced pressure. The residual oil was dried in vacuo. The yield was 21 g (92% of theory) $\eta_D^{26°} = 1.5275$, $[\alpha]_D^{25} = -3.86$ (C 5.8% w/v, in chloroform).

Thin-layer chromatography on Silica gel G with solvent A gave an R_F of 0.68.

Infrared absorption spectrum (Figure 45).

Nuclear magnetic resonance spectrum (Figure 46).

Anal. Calcd. for $C_{47}H_{67}O_3$ (676.97): C, 83.38; H, 9.53. Found: C, 83.08; H, 9.36.

C. Preparation of 2-octadecyl-3-benzyl-sn-glycerol

A solution of 14.20 g (21 mmole) of 1-O-triphenyl-2-octadecyl-3-benzyl-sn-glycerol in 100 ml of chloroform was added to 100 ml of chloroform saturated with gaseous HBr. The mixture was stirred at 0°C for 1 - 2 hours. The solvent was then removed under reduced pressure. The residue was dissolved in 25 ml of petroleum ether (b.p. 35 - 60°C) and allowed to stand overnight. The insoluble triphenylcarbinol was then removed, the supernatant was subjected to reduced pressure to remove the solvent and the residue was again dissolved in 15 ml of petroleum ether. The insoluble material was again removed. At this point, the supernatant contained most of the desired product. This supernatant was passed through a column (5 cm diamter, 200 g silicic acid, 100 mesh, 100 g celite) in hexane. The column was washed with 500 ml of hexane, 3 liters of 2 % diethyl ether in hexane, 1 liter of 2.4% diethyl ether in hexane, 1.6 liter of 3.5% diethyl ether in hexane and 400 ml of 4.0% diethyl ether in hexane to remove unhydrolyzed material and triphenylcarbinol. Then the column was washed with 4 liters of 5% - 15% diethyl ether in hexane to remove the desired compound. The fractions were combined and the solvent was

Figure 45. Infrared absorption spectrum of 1-O-triphenylmethyl-2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophometer.

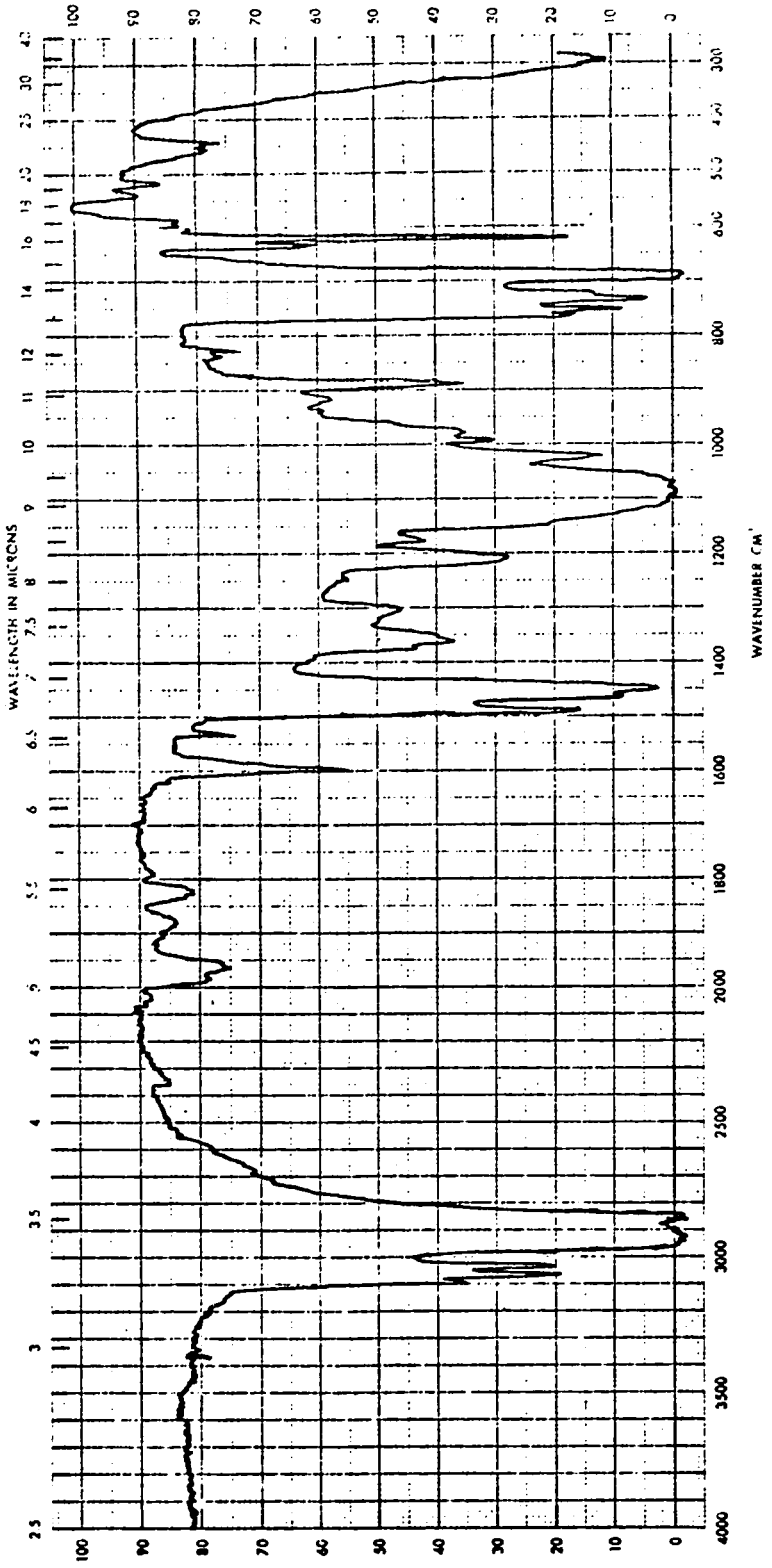
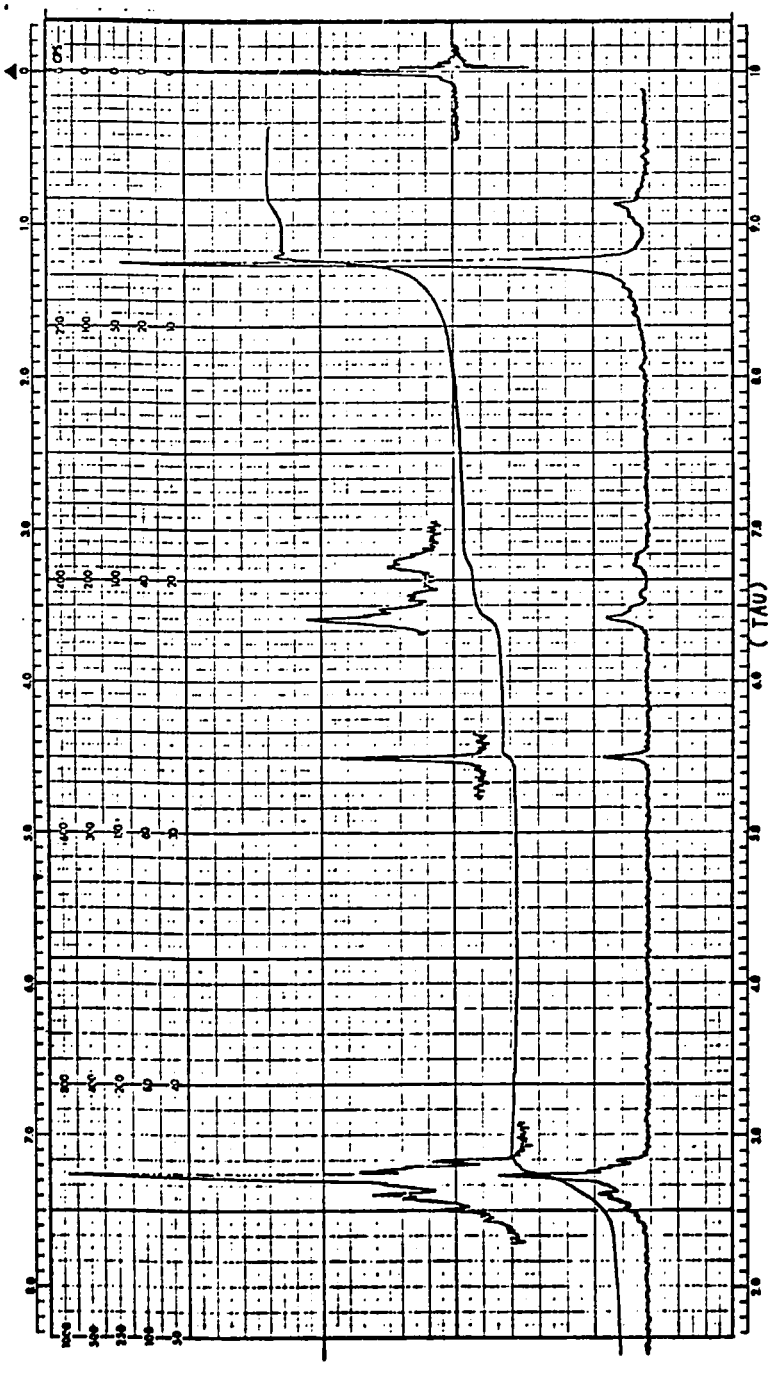

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 46. Nuclear magnetic resonance spectrum of 1-O-triphenylmethyl-2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


δ
(TAU)

removed under reduced pressure. The residual oil was dried in vacuo overnight. The yield was 7.2 g (80% of theory). $[\alpha]_D^{25}$ + 8.7 (C 6% w/v, in chloroform).

Thin-layer chromatography on silica gel G with solvent A gave an R_F of 0.15.

Infrared absorption spectrum (Figure 47).

Nuclear magnetic resonance spectrum (Figure 48).

Anal. Calcd. for $C_{28}H_{50}O_3$ (434.68): C, 77.37; H, 11.59. Found: C, 77.76; H, 11.73.

D. Preparation of 1-cis-9'-octadecenyl-2-octadecyl-3-benzyl-sn-glycerol

To a solution of 5.8 g (13.3 mmole) of 2-octadecyl-3-benzyl-sn-glycerol in 150 ml of dry benzene was added 6 g of powdered KOH and the mixture was refluxed with stirring. The water formed was removed into a separating head. Then 5 g (14.4 mmole) of n-cis-9'-octadecenylmethane sulfonate in 40 ml of dry benzene was added through a dropping funnel. The mixture was refluxed with stirring for 42 hours. After the reaction mixture cooled to room temperature, 200 ml of water was added and the solution was extracted with 3 x 300 ml of diethyl ether. The organic layers were combined and the volume was reduced to about 600 ml under reduced pressure. Then the solution was washed successively with 2 x 300 ml of 0.5N H_2SO_4, 2 x 300 ml H_2O, 2 x 300 ml of 5% $NaHCO_3$ and 2 x 300 ml H_2O. The ether solution was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The residue was dissolved in 50 ml hexane and

Figure 47. Infrared absorption spectrum of 2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophotometer.

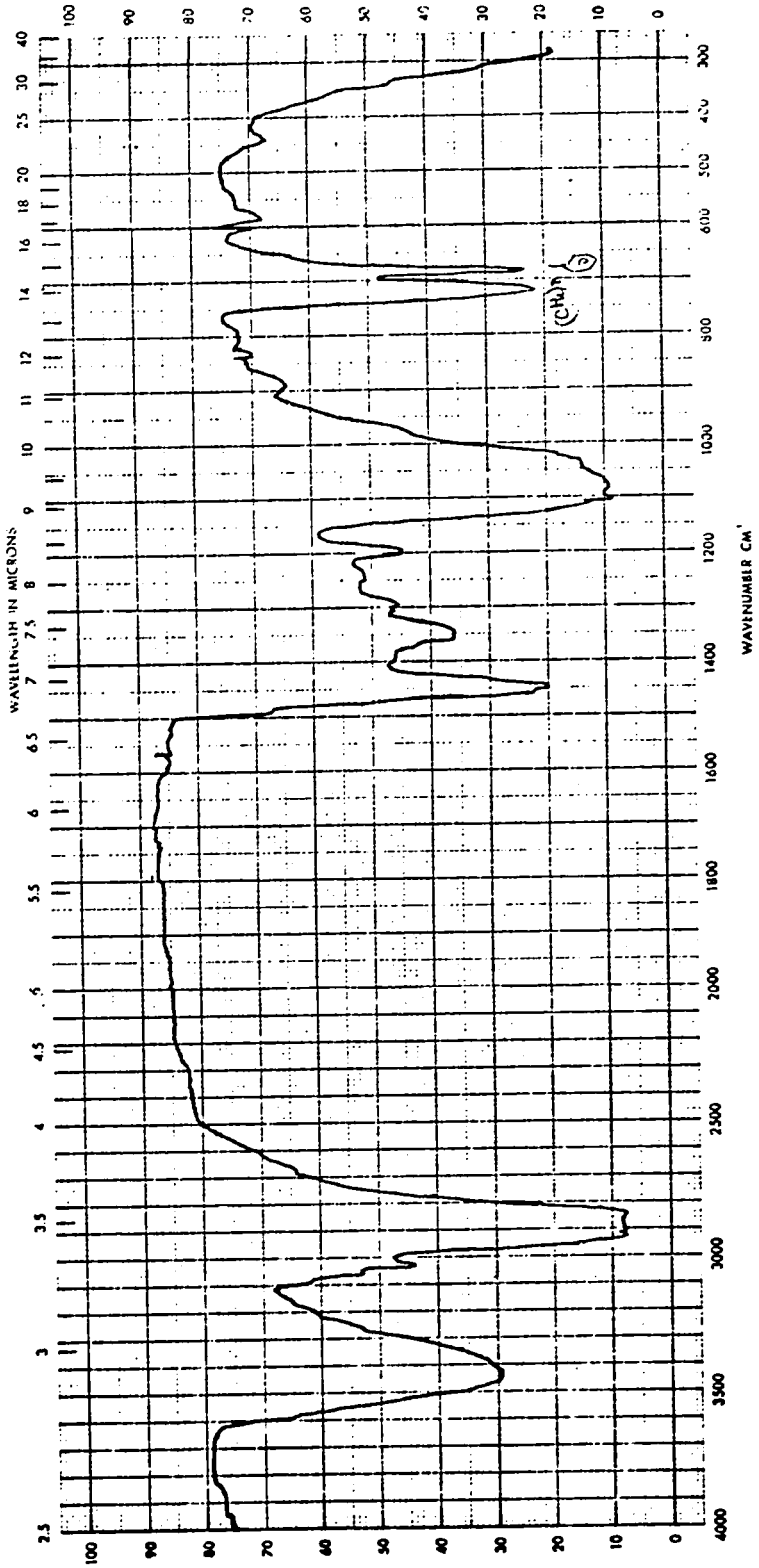
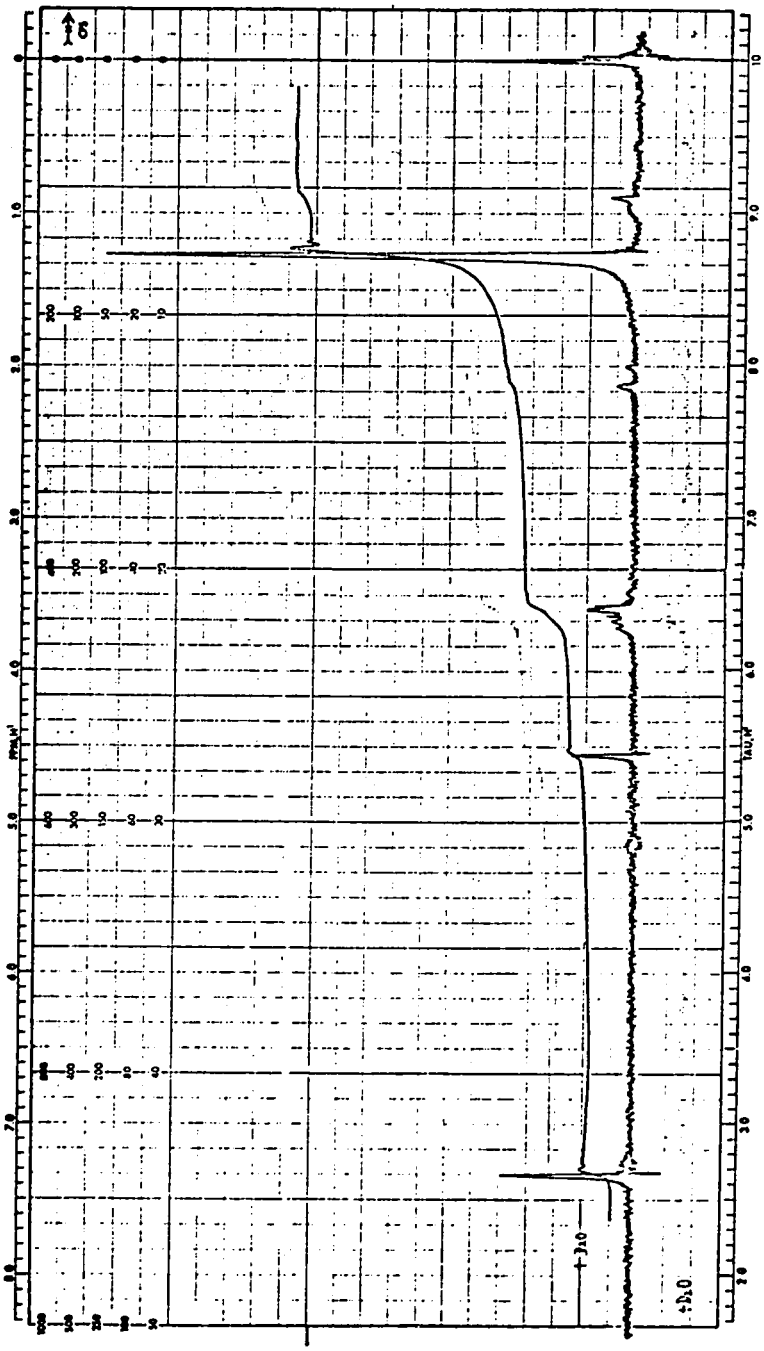

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹
(C6H5)3

Figure 48. Nuclear magnetic resonance spectrum of 2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.

L



passed through a column (5 cm diameter, 150 g Silic ARCC-7, 75 g celite) in hexane. The column was first washed with 750 ml of hexane, 750 ml of 0.1% diethyl ether in hexane and 2 liters of 1% diethyl ether in hexane to remove the fast moving components, then with 1.7 liter of 1.2% diethyl ether, 400 ml of 1.7% diethyl ether and 1 liter of 2% diethyl ether in hexane to wash off the desired compound. The appropriate fractions were combined and the solvent was removed under reduced pressure. The residue was dried in vacuo. The yield was 7.8 g (85% of theory). $n_D^{26.5°C} = 1.4746$ $[\alpha]_D^{25} - 0.72$ (C, 6.2% w/v, in chloroform).

Thin-layer chromatography on silica gel G with solvent A gave an R_F of 0.85.

Infrared absorption spectrum (Figure 49).

Nuclear magnetic resonance spectrum (Figure 50).

Anal. Calcd. for $C_{46}H_{84}O_3$ (685.13): C, 80.64; H, 12.36. Found: C, 80.90; H, 12.07.

E. Bromination of 1-cis-9'-octadecenyl-2-octadecyl-3-benzyl-sn-glycerol

In a 500 ml two-necked flask equipped with a magnetic stirrer, dropping funnel and calcium chloride tube, was placed a solution of 6.6 g (9.6 mmole) of 1-cis-9'-octadecenyl-2-octadecyl-3-benzyl-sn-glycerol in 150 ml of petroleum ether (b.p. 35 - 60°C). The flask was immersed in an ice-salt bath at -16° ± 2°C and a solution of 3.3 g (20 mmole) of bromine in 25 ml of petroleum ether was added, with stirring, over a period of 2 hours. The cold mixture was washed immediately with 2 x 150 ml of 0.5N $Na_2S_2O_3 \cdot 5H_2O$ solution, 2 x 150 ml

Figure 49. Infrared absorption spectrum of 1-cis-9'-octadecenyl-2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophotometer.

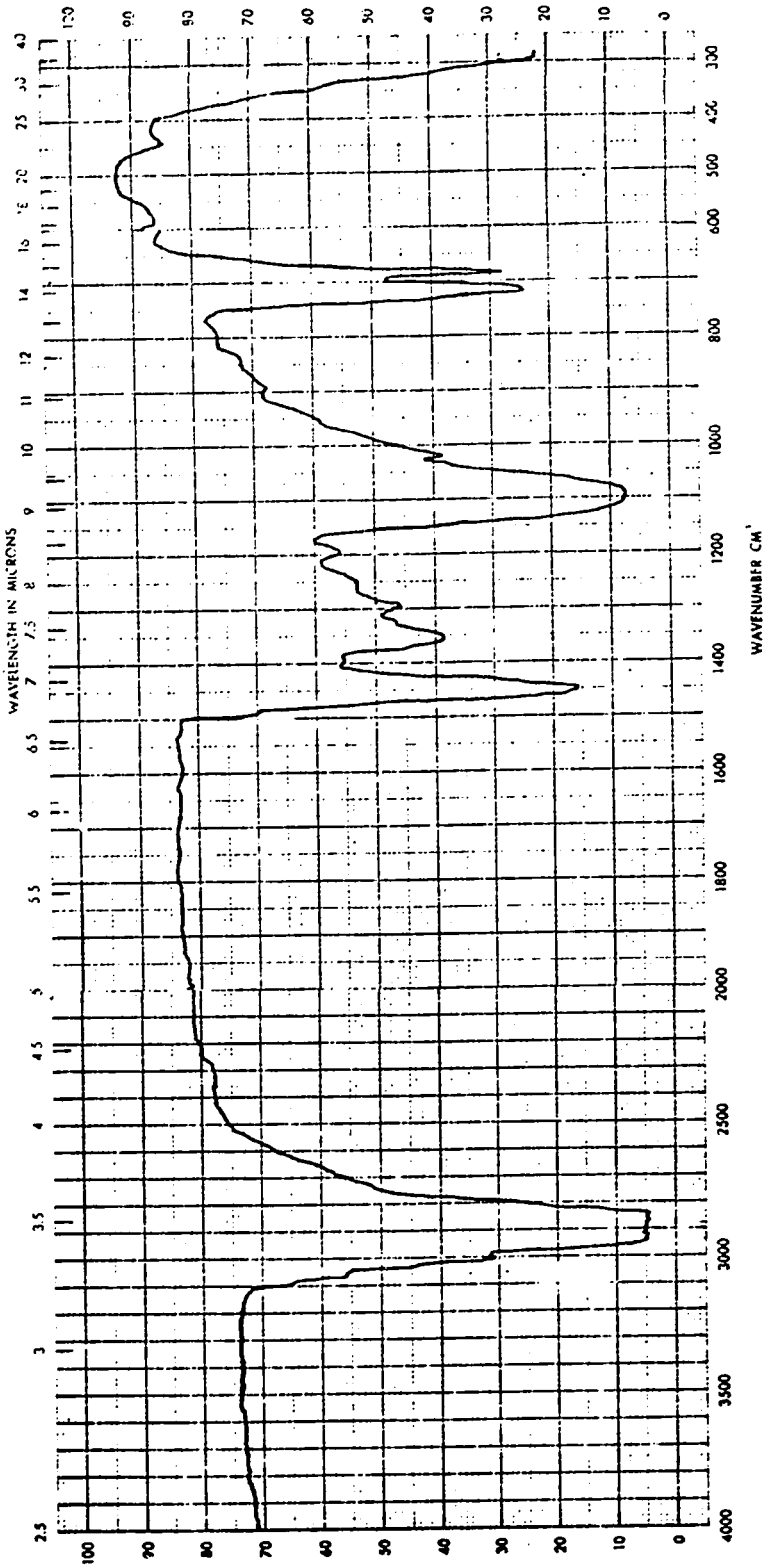

WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 50. Nuclear magnetic resonance spectrum of 1-cis-9'-octadecenyl-2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.

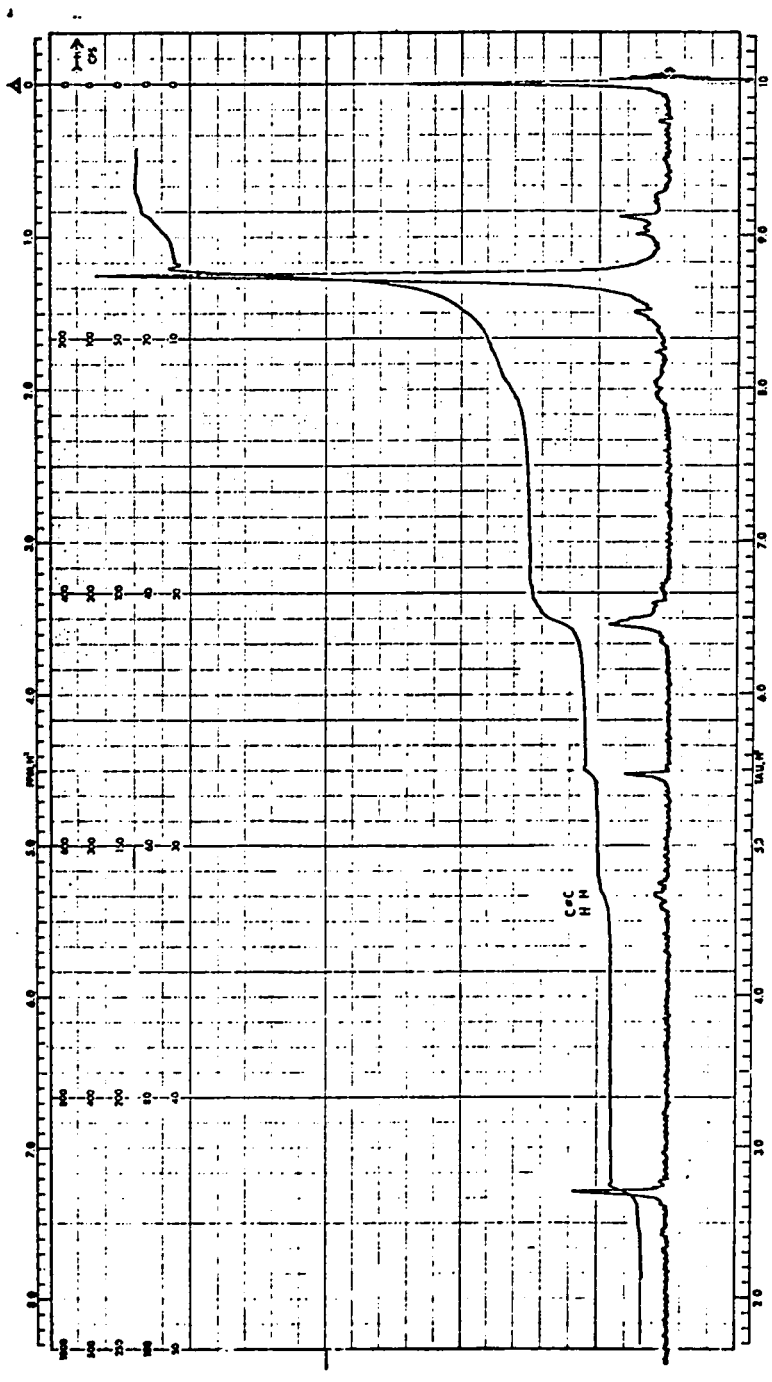
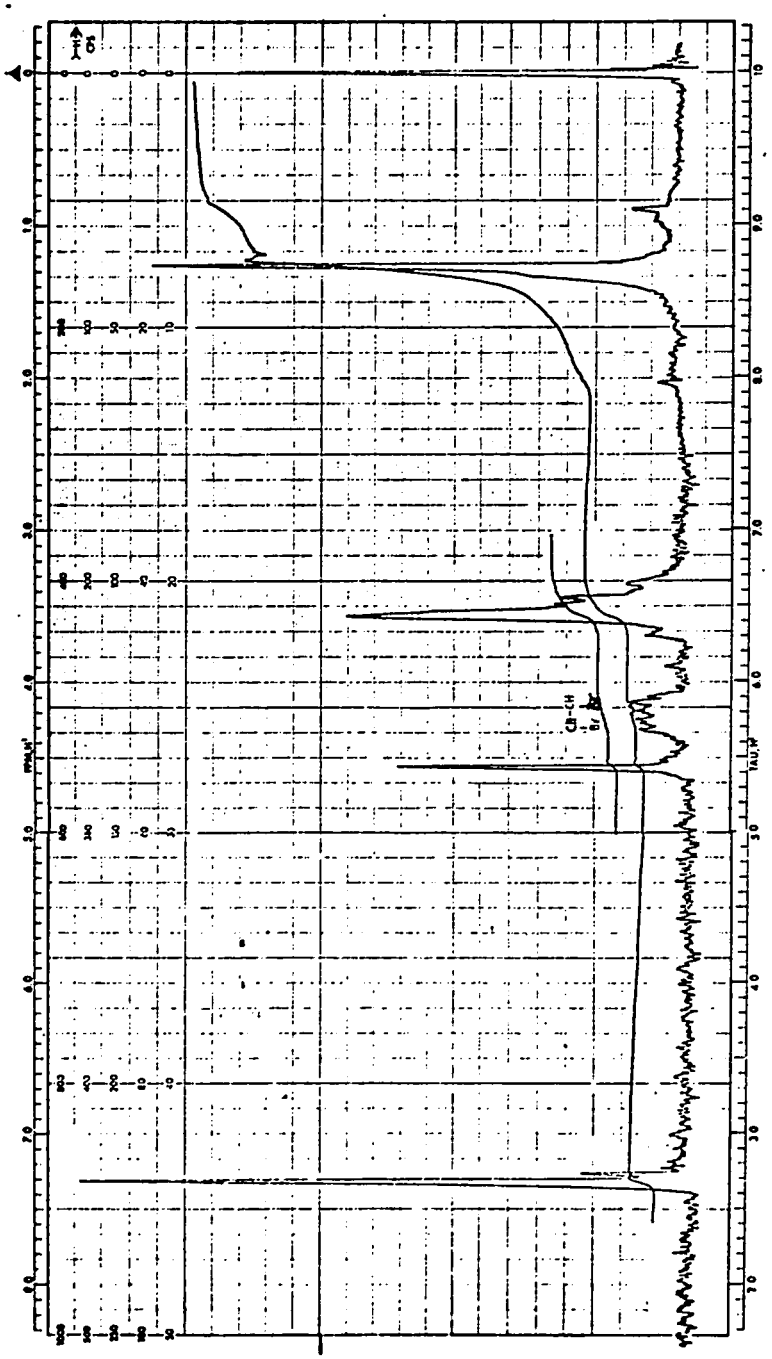

C=C
H N

Figure 51. Nuclear magnetic spectrum of 1-(Bis-9,10-cis-dibromo)octadecyl-2-octadecyl-3-benzyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


δ
CH-CH
Br
Br

of 5% $KHCO_3$ and 2 x 150 ml of distilled water. The petroleum ether solution was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The residue was dried in vacuo. The yield was 7.1 g (crude product). The nuclear magnetic resonance spectrum showed that the double bond had been lost and that $-\underset{Br}{CH}-\underset{Br}{CH}-$ had appeared (Figure 51).
This compound was not further purified and was subjected to hydrogenolysis to remove the benzyl protecting group.

F. Preparation of 1-(Bis-9,10-cis-dibromo) octadecyl-2-octadecyl-sn-glycerol

A solution of 4.0 g (4.7 mmole) of the above compound in 80 ml of ethyl acetate was added to 0.5 g of freshly prepared palladium on charcoal catalyst (152). Hydrogenolysis was carried out in an atomosphere of H_2 at room temperature and an initial pressure of about 21 p.s.i. After the reaction was complete, the catalyst was removed by filtration and washed with chloroform and the combined filtrates were evaporated to dryness under reduced pressure. The residual oil was then dissolved in 10 ml of hexane and chromatographed on a column (2 cm diameter, 120 g Silic ARCC-7, 60 g celite) in hexane. The column was first washed with 2.5 litersof 2% diethyl ether to remove a fast moving component and then with 2.8 litersof 5% diethyl ether in hexane to remove the desired product. The desired fractions were combined and the solvent was removed under reduced pressure. The residual oil was dried in vacuo. The yield was 3.0 g (86% of theory). n_D^{25} = 1.4793. $[\alpha]_D^{25}$ = -4.9 (C 7.5% w/v, in chloroform).

Thin-layer chromatography on Silica gel G with solvent A gave an R_F of 0.16.

Infrared absorption spectrum: (Figure 52).

Nuclear magnetic resonance spectrum: (Figure 53).

Anal. Calcd. for $C_{39}H_{78}O_3Br_2$ (754.83): C, 62.05; H, 10.42; Br, 21.17. Found: C, 62.69; H, 10.28; Br, 21.20.

Figure 52. Infrared absorption spectrum of 1-(Bis-9,10-cis-dibromo)octadecyl-2-octadecyl-sn-glycerol. The spectrum was taken as the $CHCl_3$ solution using an IR 10 spectrophotometer.

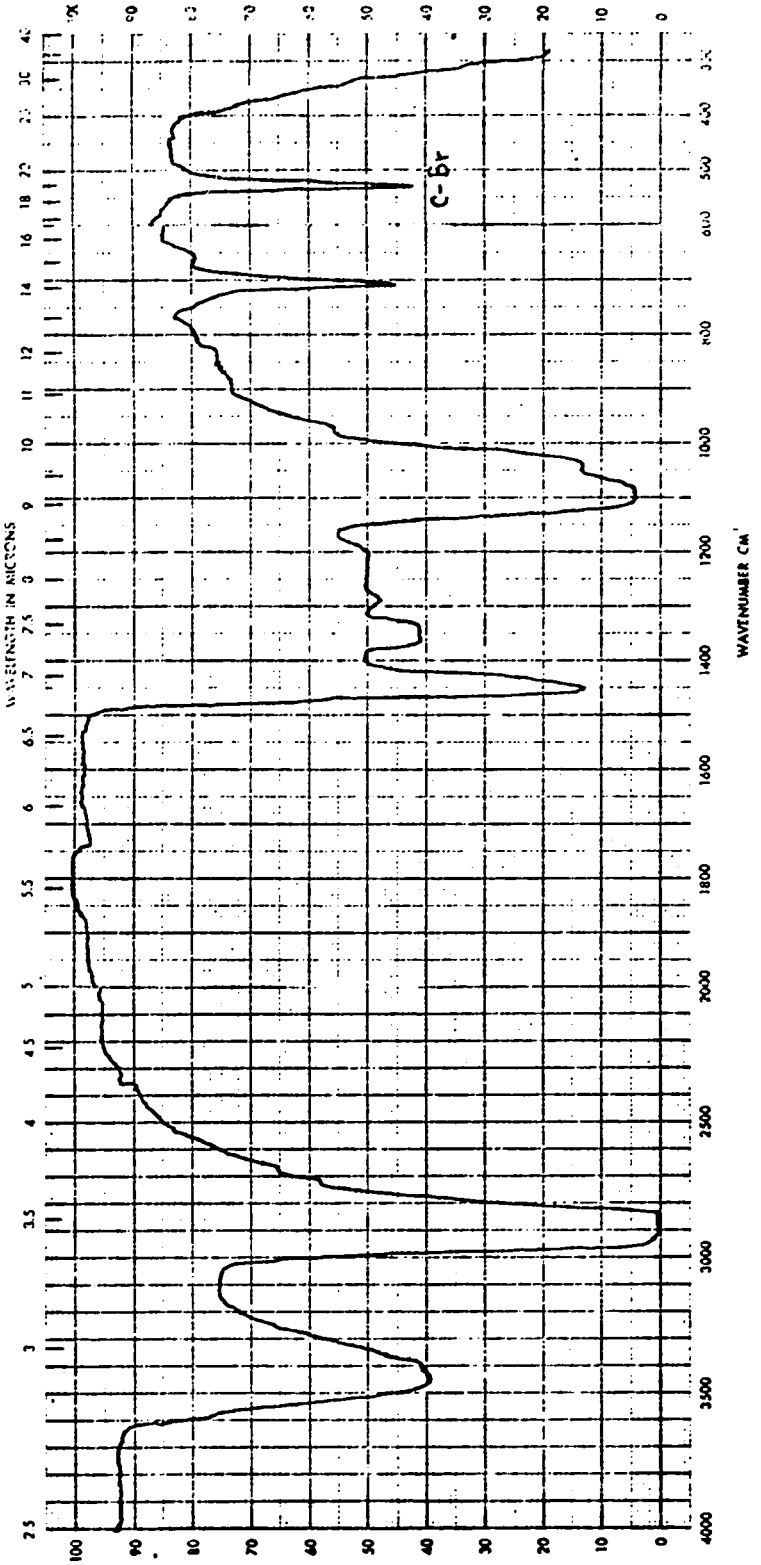
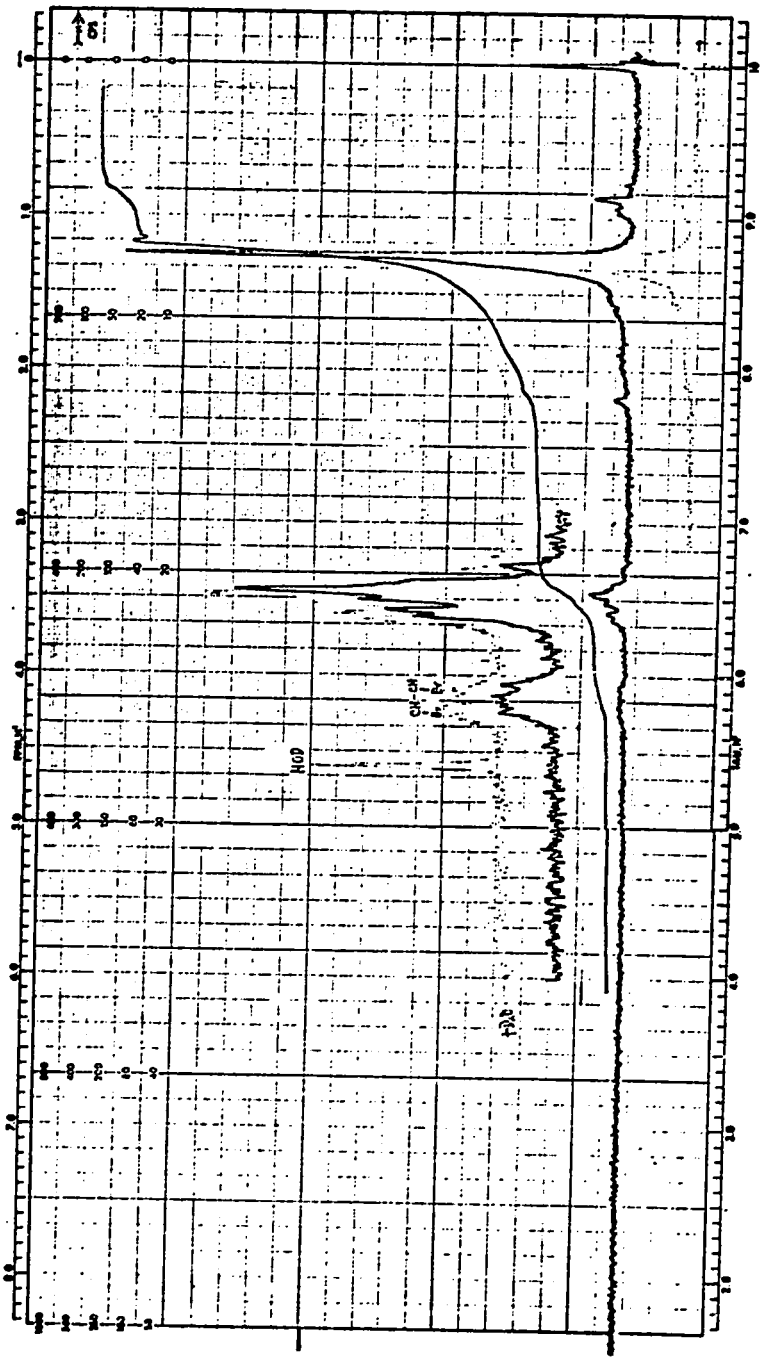

C- br
WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹

Figure 53. Nuclear magnetic spectrum of 1-(Bis-9,10-cis-dibromo)octadecyl-2-octadecyl-sn-glycerol. The spectrum was taken as the $CDCl_3$ solution using a Varian A-60 instrument.


δ
HOD