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CHEMICAL AND PHYSICAL STUDIES OF PHOSPHATIDYL SULFOCHOLINE, A SULFONIUM ANALOGUE OF PHOSPHATIDYL CHOLINE.

by

Paul-Alain Tremblay

A thesis
presented to University of Ottawa
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
Biochemistry

OTTAWA, Ontario, 1982

To my dearest mother

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ABSTRACT

A sulfonium analogue of lecithin has been reported to replace the ubiquitous phosphatidyl choline in a non-photosynthetic diatom, Nitzschia alba. The structure of this sulfonium analogue has now been established by chemical synthesis using the following methods: (i) condensation of sn-3-phosphatidic acid (dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl-) with sulfocholine (chloride or tosylate) in the presence of triisopropylbenzenesulfonyl chloride in chloroform-pyridine (9:1, v/v); and (ii) phosphorylation of 1,2-dipalmitoyl-sn-glycerol with monophenyl phosphoryl dichloridate followed by a reaction with sulfocholine in the presence of pyridine and finally removal of the blocking phenyl group by catalytic hydrogenolysis. The desired synthetic products were obtained in overall yields of 50-70 % and 11 % for methods (i) and (ii), respectively, and were characterized by elemental analyses; infrared spectroscopy; nuclear magnetic resonance spectrometry; mass spectrometry; optical rotation; and thin-layer chromatography mobilities. Comparison of the synthetic analogues with the natural sulfolecithin showed them to be identical, except for the nature of the fatty acid chains, thus establishing the natural product as sn-3-phosphatidyl sulfocholine.

Having synthesized the above PSCs, we then studied the physical properties of these amphipaths in aqueous media as compared to those of PC using the following techniques: differential scanning calorimetry (DSC), fluorescent polarization with diphenylhexatriene (DPH), para-quaterphenyl (Q-4) and *cis* and *trans*-parinaric acids as probes, electron paramagnetic resonance (EPR) with 5-doxyl stearic acid and N-tempostearamide as probes, fourier transform infrared spectroscopy (FTIR) and deuterium and phosphorus nuclear magnetic resonance (DMR and ^{31}PMR). Liposomal dispersions of the sulfonium analogues showed the typical multibilayer structure by electron microscopy (EM) and were in general very similar in physical behaviour to those of the corresponding PCs. However, the fully hydrated saturated PSCs consistently showed sharp main transitions 2-4 °C above those of the corresponding PCs, by all techniques used; the unsaturated PSC (di18:1-) had a transition 2-3 °C below that of di-18:1-PC and only di-14:0-PSC and di-18:1-PSC showed a well defined pretransition. Fluorescence polarization studies with *cis* and *trans* parinaric acids showed that the PSC bilayers were less ordered than the corresponding PC bilayers in both the gel and liquid crystalline states. The FTIR studies indicated that the crystal packing of the PSC chains in the gel state was the same as that of the saturated PCs, while the NMR studies showed that the mobilities of the head group of PSC and PC in both the gel and liquid crystalline

states are similar. Finally the monolayer studies demonstrated that PSC head groups are more interactive than PC head groups and that both derivatives have identical minimum cross sectional molecular areas. These results provide a rationale for the observed ability of the sulfonium analogues to substitute for PC in some natural membranes.

RESUME

Chez la diatomée non photosynthétique, Nitzschia alba, la phosphatidyl choline est remplacée par un analogue sulfonium de la lécithine. La structure de cet analogue sulfonium a été établie par la synthèse chimique à l'aide des méthodes suivantes: (i) condensation de l'acide sn-3-phosphatidique (dimyritoyl-, dipalmitoyl-, distéaroyl-, dioléoyl-) avec le chlorure de sulfocholine en présence du chlorure de triisopropylbenzènesulfonyl dans un mélange de chloroforme-pyridine (9:1, v/v); (ii) phosphorylation du 1,2-dipalmitoyl sn-glycérol avec le monophényl phosphoryldichloridate suivie d'une réaction avec la sulfocholine en présence de pyridine et finalement, enlèvement du groupe bloqueur phényle par hydrogénolyse catalytique. Les produits de synthèse désirés ont été obtenus avec des rendement globaux de 50 a 70 % pour la méthode (i) et 11 % pour la méthode (ii). Ces produits furent caractérisés en les soumettant aux analyses suivantes: analyse élémentaire, infrarouge, résonance magnétique nucléaire, spectrométrie de masse, rotation optique et mobilités à la chromatographie à couche mince. La comparaison des analogues synthétiques avec la sulfolécithine naturelle montre qu'ils sont identiques sauf pour la nature des chaînes d'acides gras. Le produit naturel est donc une sn-3-phosphatidyl sulfocholine.

Après avoir procédé à la synthèse des PSCs mentionnés ci-dessus, les propriétés de ces amphipathes, en ce qui a trait à leur comportement en milieu aqueux, furent comparées à celles des PCs par l'utilisation des techniques de calorimétrie différentielle à balayage, de polarisation fluorescente avec les sondes diphénylehexatriene (DPH) et les acides <u>cis</u> et <u>trans</u>-parinarique, de la résonance paramagnétique des électrons avec l'acide 5-doxyle stéarique et l'amide N-tempostéarique comme sondes, de la spectroscopie infrarouge de transformation de fourier (IRTF) et de la résonance magnétique nucléaire du deutérium et du phosphore (RMD, RM^{31}P). Les dispersions aqueuses des analogues sulfonium montrèrent la structure typique de double couches multiples (liposomes) grâce à la microscopie électronique (ME). L'on put conclure qu'ils avaient en général un comportement physique très semblable à ceux des PC correspondants. Cependant, les résultats de toutes les techniques démontrèrent que la température de transition des multidouble couche de PSC saturés en milieu aqueux accusa une courbe aiguë de 2 à 4 °C au-dessus de la température des PC correspondants. Le PSC non saturé di-18:1-PSC accusait une température de transition de 2 à 3 °C au-dessous de celle du di-18:1-PC et seuls le di-14:0-PSC et di-18:1-PSC manifestèrent une prétransition bien définie. Les études de polarisation fluorescente avec les acides <u>cis</u> et <u>trans</u>-parinariques démontrèrent que les doubles couches de PSC étaient moins ordonnées à l'état de gel et de cristal liquide que les doubles couches de PC correspondantes. La spectroscopie IRTF indiqua que la structure cristalline des chaînes de PSC saturés à l'état de gel était la même que celle des PC saturés; tandis que la spectrométrie de l'RMD et de l'RM^{31}P démontra que la mobilité des groupes de têtes de PSC et de PC dans les états de gel et de cristal liquide était du même ordre. Enfin, les études portant sur les couches simples ont démontré que les groupes de têtes des PSC sont plus intéractifs que les groupes des têtes de PC et que les deux dérivés ont des grandeurs moléculaires minimum identiques.

Des résultats obtenus, on peut déduire que les analogues sulfonium peuvent se substituer à des PC dans certaines membranes naturelles comme ils le font, en effet, dans plusieurs organismes déjà.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

PSC, phosphatidyl sulfocholine; PC, phosphatidyl choline; 14:0-PSC, dimyristoylphosphatidyl sulfocholine; 16:0-PSC, dipalmitoylphosphatidyl sulfocholine; 18:0-PSC, distearoylphosphatidyl sulfocholine; 18:1-PSC, dioleoylphosphatidyl sulfocholine; 14:0-PC, dimyristoylphosphatidyl choline; 16:0-PC, dipalmitoylphosphatidyl choline; 18:0-PC, distearoylphosphatidyl choline; 18:1-PC, dioleoylphosphatidyl choline; SQD, sulfoquinovosyldiglyceride; TPS, triisopropylbenzene sulfonyl chloride; TLC, thin layer chromatography; QP, para-quaterphenyl; CSA, chemical shift anisotropy; DPH, 1,6-diphenylhexatriene; EPR, electron paramagnetic resonance; _sn_-3-GPC, _sn_-glyceryl-3-phosphocholine; FTIR, fourier transform infrared; Tm, main transition temperature; Tp, pretransition temperature; Ts, subtransition temperature; EM, electron microscopy; P, polarization; S, order parameter; QS, quadrupole splitting; DSC, differential scanning calorimetry.

INTRODUCTION

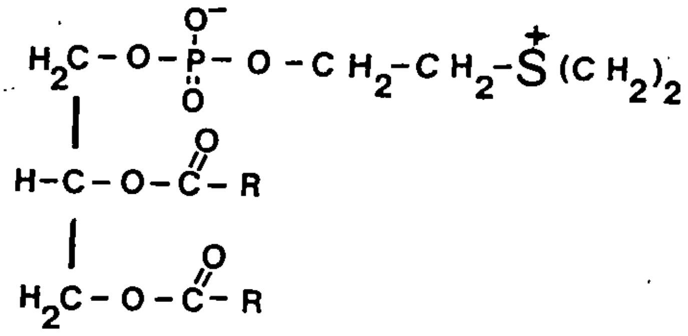
Phospholipids are a class of lipid molecules which form with protein the major constituents of biological membranes. Phosphatidyl choline (PC), the ubiquitous and most abundant phospholipid of eukaryotic membranes has an amphipathic structure with a hydrophobic tail group (diglyceride) and a polar hydrophilic head group (phosphocholine). Recently, a sulfonium analogue of phosphatidyl choline was discovered in a diatom Nitzschia alba (1). The structure of this new lipid phosphatidyl sulfocholine (PSC) is shown in comparison with that of phosphatidyl choline (PC) below (Fig. 1).

In PSC the positively charged sulfonium analog of choline, sulfocholine (S,S-dimethyl-2-thioethanol), replaces the positively charged ammonium head group of PC, choline. Both PC and PSC are zwitterionic and neutral at pH 7 since the phosphate bears a negative charge and the positively charged trimethyl ammonium or dimethyl sulfonium group is fully ionized at this pH. The diglyceride group contains fatty acid chains (R1, R2) of varying length and degree of unsaturation. However in general the fatty acid chains at C-2 of the glycerol are unsaturated and those at C-1 are saturated. It is well established that the length and degree of unsaturation of fatty acids in the hydrophobic tail region of a phospholipid affects the gel to liquid-crystalline transition temperature and the fluidity of the bilayer structure (2). Thus, shortening the chain length and increasing the unsaturation of the fatty acid residues lowers the transition temperature and raises the fluidity of the membrane bilayers (87). However membrane fluidity is also affected by the nature of the polar head group (87).

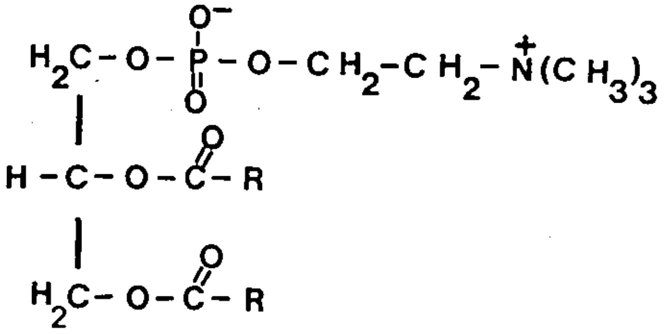
The existence of a sulfonium analogue of PC in a natural membrane raised the question concerning the effect of the sulfonium group on physical and bilayer properties of lecithin. The synthesis of PSC was then undertaken to provide sufficient material for such a study, as well as providing final proof of the structure of PSC. Furthermore, the question arose concerning the pathway for biosynthesis of the sulfonium analogue. In addition to the novel sulfonium analogue of PC, membranes of N. alba also contained a sterol in both free and sulfated form which was identified prior to this thesis work (3).

The research presented in this thesis is therefore divided into 2 parts as follows: The first (Part I) deals with the de novo chemical synthesis of PSC. The second (Part II) presents a study of the physical properties (fluidity and lipid polymorphism) of PC and of PSC.

Figure 1: Chemical structures of phosphatidyl sulfocholine and phosphatidyl choline. The R groups represent hydrocarbon chains



sn-3-Phosphatidyl sulfocholine



sn-3-Phosphatidyl choline

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I

CHEMICAL SYNTHESIS OF PHOSPHATIDYLSULFOCHOLINE

1.1 LITERATURE REVIEW

1.1.1 Discovery of PSC

In 1976 the non-photosynthetic marine diatom, Nitzschia alba was reported to contain phosphatidyl sulfocholine (PSC) instead of phosphatidyl choline (PC) in its biomembranes (1). The structures are illustrated in figure 1. The existence of PSC remained undiscovered for many years because of the similarity of its structure with that of PC. In fact, these two analogous lipids are so similar in solubility, charge, size and chemical properties, that they are very difficult to separate and distinguish from one the other even with modern chromatographic methods, such as TLC.

Kates and Volcani (4) grew five species of marine diatoms in the presence of ^{32}P-phosphate, ^{35}S-sulfate, and ^{14}C-acetate and the labelled lipids were visualized by autoradiography of silica gel loaded paper chromatograms. The results obtained with radioactive sulfate were quite surprising as it was found to be present in several biproducts apart from the well known chloroplast lipid sulfoquinovosyldiglyceride. One of these ^{35}S-labelled lipids, that cochromatographed with lecithin, was found in all of the five diatom species analysed. The mild alkaline hydrolysates of the lipids of these diatoms gave anomalously low amounts of the expected glycerophosphocholine. Instead, high concentrations of glycerophosphate were found. This is now known to be indicative of a high content of phosphatidyl sulfocholine and a low content of phosphatidyl choline in the five diatom species studied.

The unknown sulfur-labelled lipid was identified ten years later in the diatom <u>Nitzschia alba</u> by Anderson <u>et al</u> (1, 5, 6) who also identified two other novel sulfolipids in <u>N. alba</u>. The isolation and identification of the hitherto unknown phosphatidyl sulfocholine was made possible by the fact that they chose an organism <u>N. alba</u> that happened not to contain any phosphatidyl choline in its membranes. Thus, the problem of separating PSC from PC did not arise and the isolation of pure PSC was relatively easily achieved.

The identification of PSC was accomplished as follows. The lipid cochromatographed with lecithin and had a phosphorus to sulfur ratio of 1 to 1. Proton NMR spectra showed all the characteristic signals of lecithin, except that a singlet at 3.17 δ assigned to sulfonium methyl groups replaced the ammonium methyl singlet at 3.37 δ. Infrared spectra showed the absence of a band at 970 cm^{-1} present in the spectrum of PC, and assigned to the quaternary ammonium group.

Enzymatic degradation gave the expected products (Fig. 2): phospholipase D gave phosphatidic acid and sulfocholine, while phospholipase C gave diglyceride and phosphosulfocholine. Chemical hydrolysis of PSC with 2.5% methanolic HCl gave sulfocholine, glycerophosphate and fatty acid methyl esters (the expected products by analogy with lecithin), but mild alkaline deacylation with methanolic NaOH gave glycerophosphate and methoxysulfocholine instead of the expected glycerophosphorosulfocholine (see above). The PSC readily undergoes attack by a methoxy ion to give methoxy sulfocholine and glycerophosphate (6) (Fig. 2). These products are characteristic of a sulfonium analogue of PC and can be used to distinguish between PSC and PC. The above results were entirely consistent with the structure of PSC as shown in Figures 1 and 3 . However, final confirmation of this structure by chemical synthesis was still required.

The structures of the other sulfur-containing lipids in N. alba (Fig. 3) were established by Anderson et al 1978 (6) with the sterol sulfate established by Kates and Tremblay (3) as follows:

1. Sulfoquinovosyldiglyceride (SQD), this lipid is a ubiquitous component of photosynthetic tissue (leaves) of higher plants (7), it is also found in some non-photosynthetic organisms including the non-photosynthetic diatom Nitzschia alba. This organism lacks a chloroplast membrane structure, but it does

however have a proplastid structure which may contain SQD.

2. <u>Ceramide sulfonate</u> (CS) an unusual lipid in which the primary OH of ceramide is replaced by a direct carbon to sulfur-bonded sulfonate group. The synthesis of this lipid was undertaken during the course of this thesis work but was abandoned when it was learned that Russian workers (8) had already published the synthesis.
3. <u>Sterol sulfate</u> (SS) This lipid was initially reported to be brassicasterolsulfate (9), but the sterol is actually the well known plant sterol, 24-methylene cholesterol, an isomer of brassicasterol (10, 11).

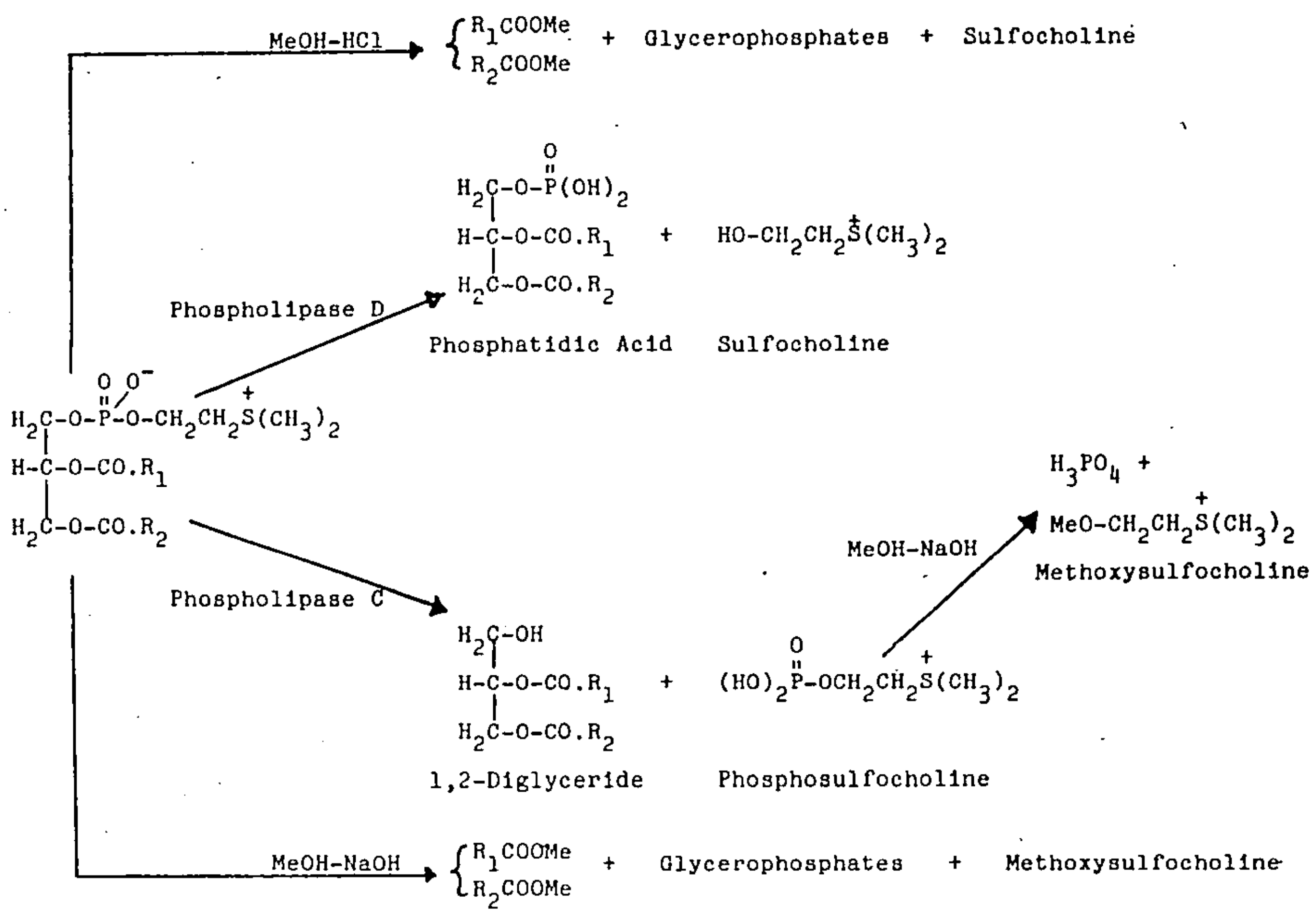
Anderson <u>et al</u> (12) then studied the metabolism of sulfolecithin. They showed that the labelled ^{35}S amino acids cysteine, cystine and methionine were effective precursors of PSC, as was inorganic sulfate. Recently Anderson and Bilan (13) demonstrated the incorporation of sulfocholine (^{35}S) into mouse LM fibroblasts with almost complete replacement of lecithin by sulfolecithin after 7 days when the cells were kept in the presence of sulfocholine as the sole choline source. This leaves little doubt that sulfocholine can act as an effective substrate in the biosynthetic pathway for lecithin.

Earlier in 1969 Bjerve and Bremer (14) fed rats with ^{3}H and ^{35}S-labelled sulfocholine in which the tritium was in the methyl groups and found that the sulfur label was incorporated into a lipid that chromatographed with lecithin. Because of a drop in the ^{3}H to ^{35}S ratio, indicating a loss of the methyl ^{3}H label relative to the ^{35}S label, no definite conclusions could be made concerning the structure of this sulfur lipid. It was not possible to separate the ^{35}S labelled lipid from the ever present PC, but they were however astute enough to propose the PSC structure for the unknown sulfur containing lipid.

Other recent studies also show the lack of specificity of the lecithin biosynthetic pathway toward choline analogues. Thus Snyder *et al* 1980 (84) found that N-isopropylethanolamine an unnatural analogue of choline competitively inhibits the incorporation of choline into phosphatidyl choline, the uptake of choline into the cell, the synthesis of DNA, RNA, protein, and the $\triangle$-9 acyl desaturase. However other membrane-bound enzyme activities such as those of NADH-cytochrome c reductase, NADPH-cytochrome c reductase etc. are unaffected. Snyder suggested that these effects are not caused by a change in fluidity of the membrane but by the alteration of choline metabolism in the cell (a change in the concentration of metabolites). Another recent study (179) involves the *in vivo* incorporation of a phosphonium analogue of choline into lecithin. ^{31}P NMR was used to monitor the incorporation of the choline analogue into lecithin. Akesson has also shown (85) in vivo methylation of various choline analogues in which the methyl groups of choline have been replaced by one and two ethyl groups. These studies show that the PC of a cell can be replaced by a number of unnatural PC analogues as a result of the broad specificity of the PC synthetase system.

To establish its structure unequivocably, the chemical synthesis of PSC was undertaken, and this in turn made available sufficient quantities of the optically pure PSC with specific fatty acids for metabolic and physical studies. The approach used was similar to that for the synthesis of PCs and in the following sections the procedures that have been developed will be reviewed (15, 16, 17, 18, 19, 52).

Figure 2: Hydrolytic reactions used by Anderson et al (7) for the elucidation of the structure of phosphatidyl sulfocholine from the diatom *Nitzschia alba*.

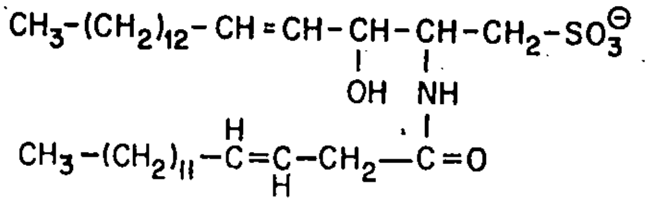


Scheme 2. Hydrolytic reactions used for elucidation of the structure of phosphatidylsulfocholine.

Figure 3: The chemical structures of the four novel sulfolipids from the diatom Nitzschia alba.

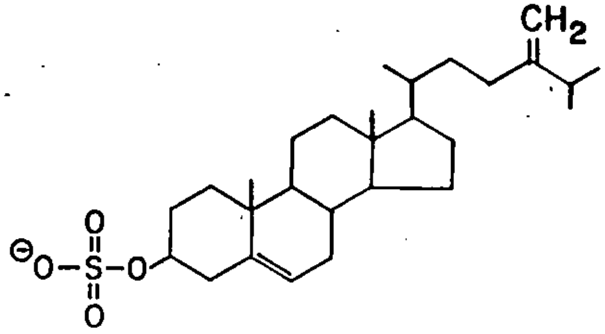
SULFOLIPIDS of *N. alba*

N-acyl-1-deoxysphingenine-1-sulfonate

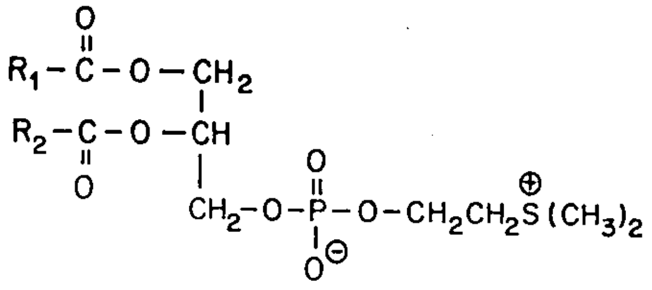


$$\begin{array}{l} CH_3-(CH_2)_{12}-CH=CH-\underset{OH}{\underset{|}{CH}}-\underset{NH}{\underset{|}{CH}}-CH_2-SO_3^{\ominus} \\ CH_3-(CH_2)_{11}-\overset{H}{C}=\underset{H}{C}-CH_2-\overset{|}{C}=O \end{array}$$

24-Methylene cholesterol sulfate

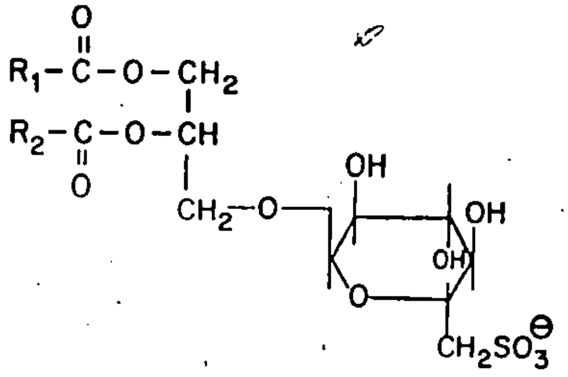


Phosphatidyl S,S-dimethyl mercaptoethanol



$$\begin{array}{l} R_1-\overset{O}{\overset{\|}{C}}-O-CH_2 \\ R_2-\underset{O}{\underset{\|}{C}}-O-\overset{|}{CH} \\ \qquad\qquad\quad \overset{|}{CH_2}-O-\overset{O}{\overset{\|}{P}}\underset{O^{\ominus}}{\underset{|}{}}-O-CH_2CH_2\overset{\oplus}{S}(CH_3)_2 \end{array}$$

Sulfoquinovosyl diglyceride



1.1.2 Chemical Synthesis of Phosphatidyl Cholines

1.1.2.1 Saturated PCs

Baer and Kates in 1950 (20) first reported the synthesis of enantiomeric forms of lecithin. The synthesis involved phosphorylation of a saturated sn-1,2-diglyceride with monophenyl phosphoryldichloridate in the presence of quinoline and the subsequent reaction with anhydrous choline chloride in the presence of pyridine to yield phenyl-1,2-diacyl-sn-glyceryl-3-phosphoryl choline which upon hydrogenolysis of the phenyl group gave in low yield the first fully saturated series of sn-3-phosphatidyl cholines (dimyristoyl, dipalmitoyl, and distearoyl) (Fig. 4). Subsequently an improved and simplified procedure was reported by Baer and Marukas (21) for the synthesis of saturated lecithins including lecithin containing water-soluble short chain fatty acids.

1.1.2.2 Unsaturated PCs

Because hydrogenolysis is used to remove the protecting phenyl group, the synthesis of unsaturated lecithins by the above route is not possible. Baer, Buchnea and Newcombe (22) reported the first synthesis of a fully unsaturated sn-3-phosphatidyl cholines by a long route involving phosphorylation of 1,2-isopropylidene-sn-glycerol with monophenylphosphorylchloridate followed by reaction with 2-chloroethanol in pyridine to yield phenyl-2'-chloroethyl-1,2-isopropylidine-sn-glyceryl-3-phosphate from which the phenyl and isopropyl protecting groups were removed by hydrogenolysis and acid hydrolysis respectively. The free hydroxyls of 2-chloroethylphosphoglycerol were then acylated with oleoyl chloride in the presence of pyridine. The resulting dioleoyl glycerophosphorylethyl chloride was reacted with trimethylamine to give 1,2-dioleoyl sn-glycero 3-phosphoryl choline. This last step is similar to the procedure for lecithin synthesis reported later by Hirt and Birchhold (23) and then improved by Eible et al (24). They condensed diglyceride with bromoethyldichlorophosphatidate and isolated 2-bromoethylphosphatidate which was converted to lecithin by substituting the bromide with trimethylamine, with an overall yield of 57%.)

Baer and Kindler (25) synthesized unsaturated lecithins using a much simpler approach. They condensed phosphorus oxychloride with 1,2-dioleoyl-sn-glycerol in the presence of quinoline. The resulting dichloride derivative of phosphatidic acid was esterified with choline iodide. This method leads to many biproducts but the desired product was purified using silicic acid column chromatography.

Several methods have been reported for the synthesis of both saturated and unsaturated homogeneous lecithins that use glycerophosphocholine (37, 36, 30, 31, 29, 28, 27, 26)

(Fig. 5). The methods differ from one another generally in the acylating agents, the organic bases or the solvents used. The starting material sn-3-GPC is conveniently obtained by mild alkaline hydrolysis of egg lecithin with a suitable base such as tetra-n-butyl ammonium hydroxide (34, 33, 32, 35).

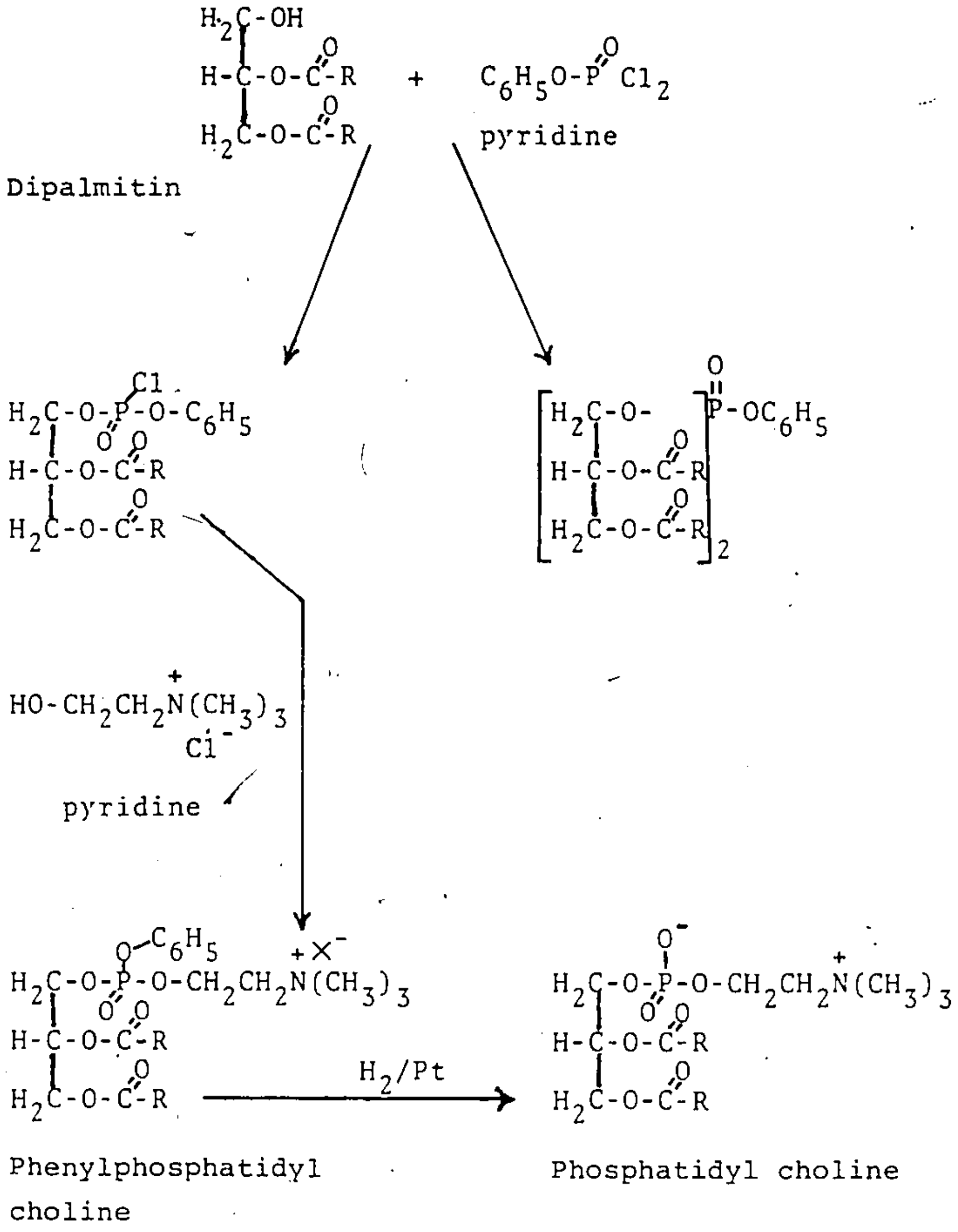
Tattrie and McArthur (26) reported the use of fatty acid chlorides to acylate sn-3-GPC in anhydrous chloroform. Kögl et al (27) synthesized other derivatives of lecithin by this method and Baer and Buchnea (28) improved on the yield by using the cadmium chloride adduct of sn-3-GPC and pyridine as the base. Cubero Robles and van den Burg in 1969 (29) reported the synthesis of lecithin by acylation of sn-3-GPC with fatty acid anhydride and the sodium salt of the fatty acid in a sealed tube at elevated temperatures (70°C). This method gives poor yields if saturated fatty acids are used. Pugh and Kates (30) synthesized unsaturated lecithins with the activated mixed anhydride of the fatty acid and trifluoroacetic acid in the presence of pyridine and anhydrous chloroform.

More recently methods have been published using more polar solvents and more active bases with much shorter reaction times. Thus Warner and Benson (36) have greatly improved the yields with a very fast reaction in a reaction mixture containing fatty acid imidazole and the very strong non-nucleophilic base: sodium dimethylsulfoxide carbanion. Unfortunately migration of the phosphate ester groups sometimes occurs in the strongly basic solution with the concurrent production of 1,3-diacyl-sn-2-glycerophosphocholine if the reagents are not mixed immediately following dissolution of the sn-3-GPC (personal discovery). Another method giving a high yield of product was recently reported by Patel, Morisset and Sparrow (37); sn-3-GPC is acylated in the following reaction mixture consisting of: the base 4-N-pyrrolidinopyridine and fatty acid anhydride in the polar aprotic solvent mixture dimethyl sulfoxide/benzene (1,1, v/v).

1.1.2.3 Method of Aneja

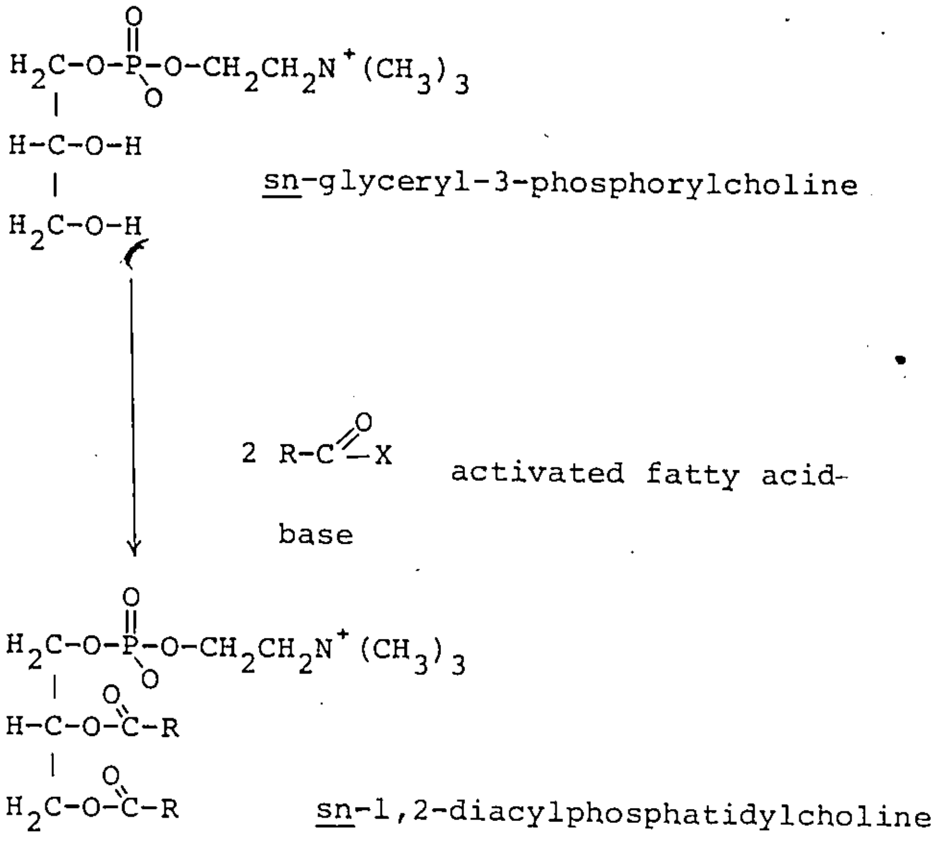
A novel synthesis of PC was published by Aneja and Chadha (38). This synthesis used the coupling agent triisopropylbenzenesulfonyl chloride (TPS) to couple the two starting materials phosphatidic acid and choline chloride in the presence of choline at 70 °C. This temperature is too high for the synthesis of certain of the (poly) unsaturated lecithins, due to the risk of auto-oxidation. Because of its bulky isopropyl groups, TPS will only react with free and singly substituted phosphate groups to form sulfonate-phosphate anhydrides and thus prevents the synthesis of lecithin with two choline groups. The arylsulfonate is also an excellent leaving group. The product is obtained in a high yield of approximately 75% (Fig. 6).

Figure 4: The first chemical synthesis of phosphatidyl choline as reported by Baer and Kates (20).


Dipalmitin
pyridine
HO-CH2CH2N(CH3)3 Cl-
pyridine
H2/Pt
Phenylphosphatidyl choline
Phosphatidyl choline

R = $CH_3(CH_2)_{14}$-

Figure 5: Partial chemical synthesis of phosphatidyl choline from <u>sn</u>-glyceryl-3-phosphocholine.

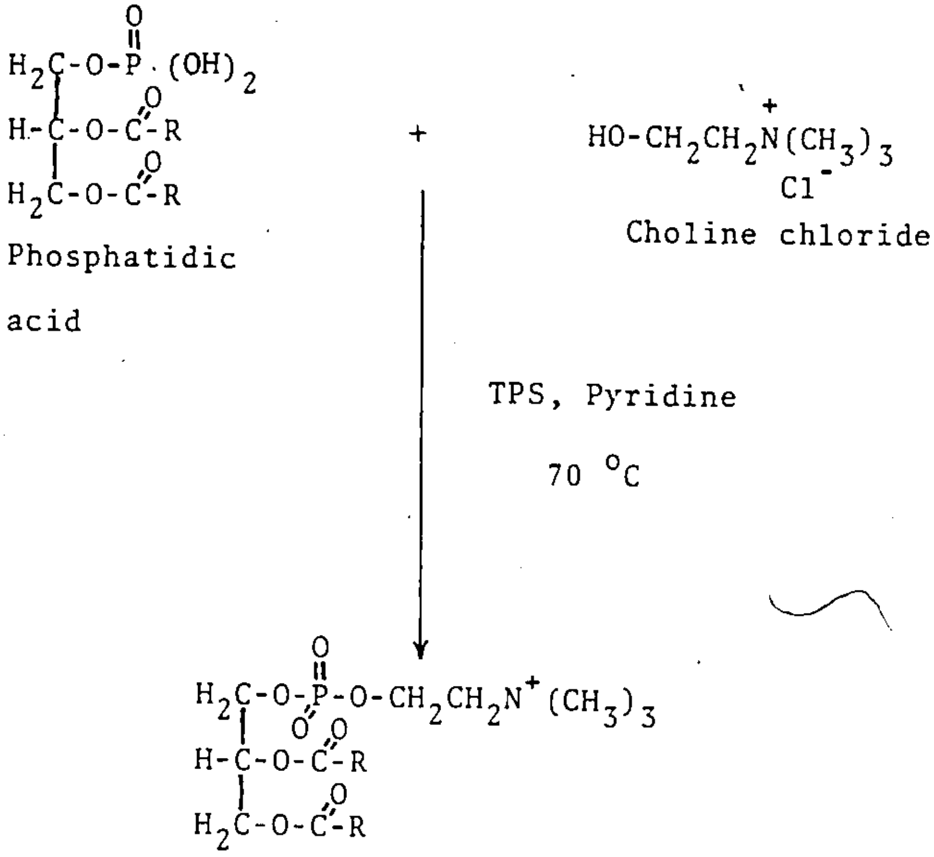


R= saturated of unsaturated chains

X= chloride, RCO-O-, trifluoroacetate, imidazolide, etc.

base= pyridine, fatty acid salt, sodium dimethylsulfoxide carbanion, etc.

Figure 6: The chemical synthesis of phosphatidyl choline by the method of Aneja and Chadha (38).



Phosphatidyl choline

R = saturated or unsaturated chains

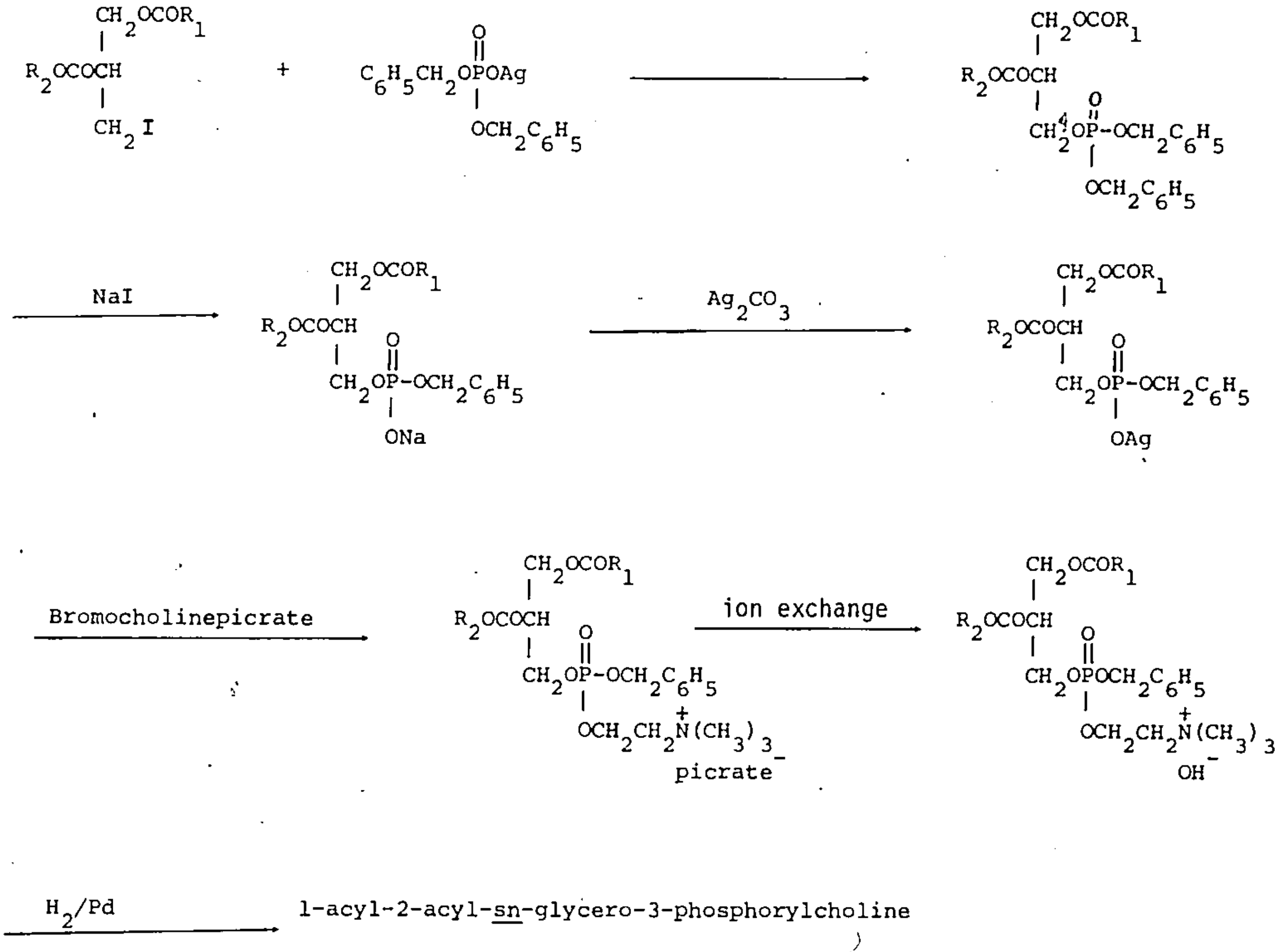
1.1.2.4 Mixed fatty acid PCs

De Haas and Van Deenen (39) were the first to synthesize *de novo* mixed fatty acid lecithins. The starting material 1,2-diacyl-*sn*-glycerol-3-iodohydrin was first phosphorylated with dibenzylchlorophosphate and then monodebenzylated to the sodium salt of benzylphosphatidic acid. The sodium-salt was then converted to the silver salt, which was then reacted with bromocholine picrate giving benzyl lecithin; finally this product was debenzylated by hydrogenolysis to 1,2-diacyl-*sn*-glycero 3-phosphoryl choline (Fig. 7).

More recently mixed acid lecithins have been synthesized by many authors (40, 29, 41, 42, 43, 44, 45) from monoacid lecithins (synthetic) via 1-acyl-*sn*-glycero-3-phosphoryl choline. The lyso-compound can be conveniently prepared by the action of phospholipase A-2 on the monoacid lecithin. The free 2-hydroxyl of lysolecithins can be reacylated by one of the methods described in subsection 1.3.2

The method of Cubero Robles and van den Berg (29) could not be used for synthesis of PSC, since glycerophosphoryl sulfocholine was unstable under the alkaline conditions involved, an alternative method using sulfocholine had to be considered. The method of Aneja and Chadha (38) seemed more promising provided lower temperatures could be used. In order to test this sulfocholine was required and its chemical synthesis will now be reviewed.

Figure 7: The chemical synthesis of mixed acid phosphatidyl cholines by the method of van Deenen (52)


NaI
Ag$_2$CO$_3$
Bromocholinepicrate
ion exchange
picrate
H$_2$/Pd
1-acyl-2-acyl-sn-glycero-3-phosphorylcholine

1.1.3 Chemical Synthesis of Sulfocholine

Sulfocholine was recognised early as a sulfur analog of choline by Renshaw et al 1926 (46). Sulfocholine or S,S-dimethyl-2-hydroxylethylsulfonium chloride as it is systematically called, was first prepared as the iodide by the reaction of ethylene iodohydrin with dimethyl sulfide over a period of several days. The product, sulfocholine iodide, was not pure, however, due to the dissociation of sulfocholine to methyl iodide and methylthioethanol and subsequent reaction of the methyl iodide with dimethyl sulfide to give trimethylsulfonium iodide. Many other side products, for example methyl-di-2-hydroxyethyl sulfonium iodide, are formed by analogous reactions.

Maw and du Vigneaud 1948 (47) prepared sulfocholine iodide in two steps via S-methylthioethanol obtained from the reaction of methyl mercaptan and ethylene bromohydrin in the presence of base. Methylation of the product with methyl iodide gave sulfocholine iodide quantitatively. The chloride was obtained by treatment of a solution of sulfocholine iodide with silver chloride.

Bjerve and Bremer 1969 (14) used this method to prepare radioactive sulfocholine chloride, e.g. sulfocholine labelled in the methyl groups with tritium as well as with ^{35}S. Similarly Anderson et al (6) prepared sulfocholine iodide by the reaction of methyl iodide with mercaptoethanol for several days at room temperature. The product was purified and converted to the chloride on a column of cation exchange resin [Rexyn 101 (H^+)] by elution with increasing concentration of aqueous hydrochloric acid. The major biproduct, trimethylsulfonium chloride, was removed by this method (Fig. 8).

Because the one step methods of Renshaw <u>et al</u> (46) and Anderson <u>et al</u> (6) gave many side product, a two step approach, analogous to the method of Maw and du Vigneaud (47), was adopted in the present studies (Fig. 8). 2-Thioethanol was substituted for the foul smelling methyl mercaptan and the intermediate product, 2-methylthioethanol was purified by vacuum distillation. Final methylation of this product with methyl iodide gave sulfocholine iodide quantitatively as reported by Maw and du Vigneaud (47). The chloride was then prepared by treatment with ion-exchange resin Rexyn 101 (H^+). Radioactive sulfocholine required for the metabolic studies described in Part III was prepared by the method of Anderson <u>et al</u> because of its simplicity (Fig. 8).

Figure 8: Two procedures used for the synthesis of sulfocholine: A) a two step method, B) a direct method used for the synthesis of ^{35}S labelled sulfocholine chloride.

A

$HO\text{-}CH_2\text{-}CH_2\text{-}SH$ 2-Mercaptoethanol

$\downarrow$ KOH

$HO\text{-}CH_2\text{-}CH_2\text{-}SK$

$\downarrow$ CH_3I (1 mole)

$HO\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_3$ S-Methyl-2-thioethanol

$\downarrow$ CH_3X (excess)

$HO\text{-}CH_2\text{-}CH_2\text{-}\underset{+}{S}(CH_3)_2$ S,S-Dimethyl-2-hydroxyethylsulfonium iodide
X^-

$\downarrow$ Rexyn H^+ (101) eluted with HCl

$HO\text{-}CH_2\text{-}CH_2\text{-}\underset{+}{S}(CH_3)_2$ S,S-Dimethyl-2-hydroxyethylsulfonium chloride
Cl^-

X = tosylate, iodide

B

$HO\text{-}CH_2\text{-}CH_2\text{-}SH$ 2-Mercaptoethanol

$\downarrow$ CH_3I (excess)

$HO\text{-}CH_2\text{-}CH_2\text{-}\underset{+}{S}(CH_3)_2$ + $(CH_3)_3S^+I^-$ (biproduct) Trimethylsulfonium Iodide
I^-

$\downarrow$ Rexyn H^+ (101) eluted with HCl

$HO\text{-}CH_2\text{-}CH_2\text{-}\underset{+}{S}(CH_3)_2$ S,S-Dimethyl-2-hydroxyethylsulfonium chloride
Cl^-

1.1.4 Chemical Synthesis of Phosphatidic acids

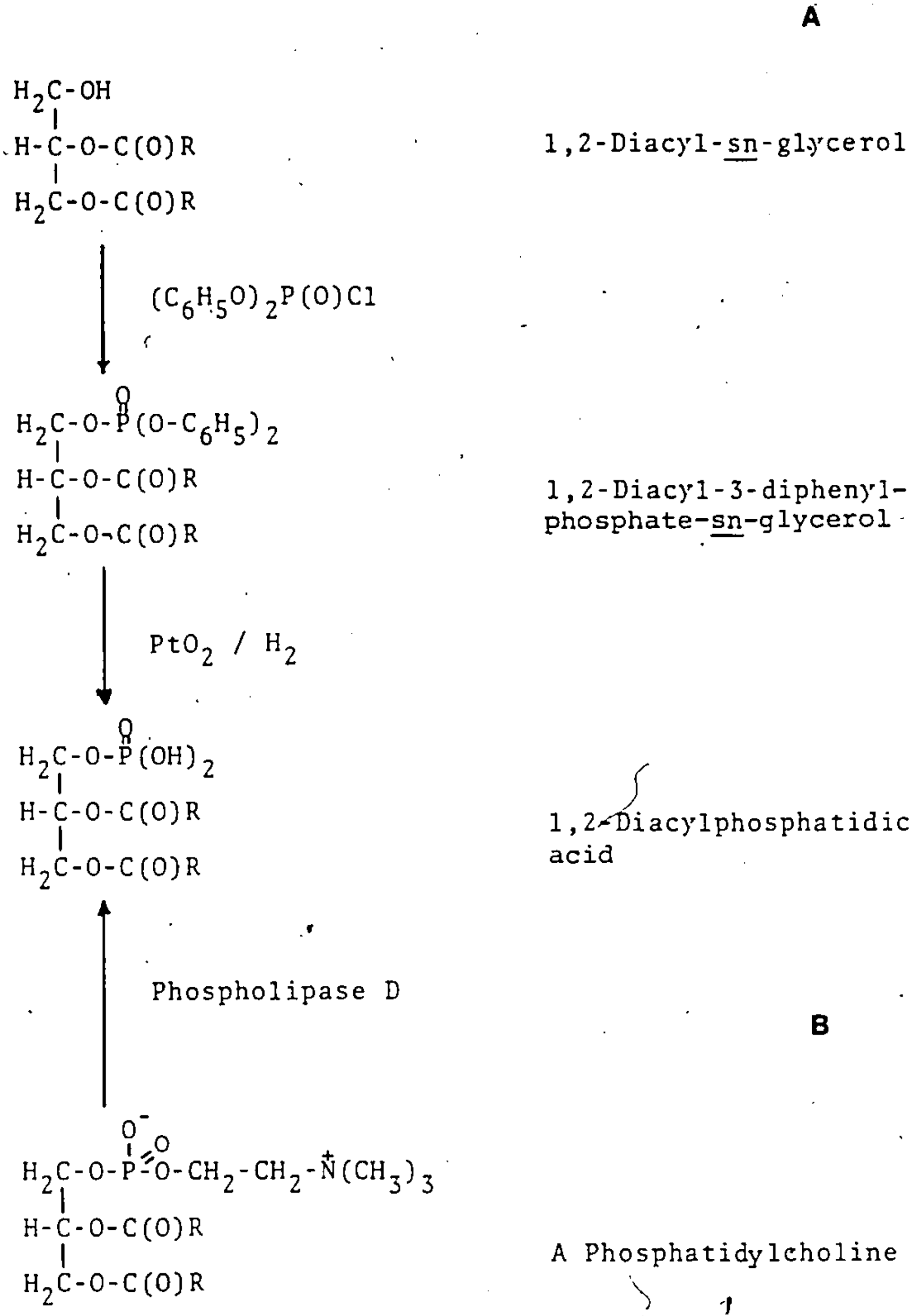
Baer (48) first reported the complete synthesis of optically pure phosphatidic acids with monoacid saturated fatty acid side chains using the condensation of diphenylphosphorylchloride with sn-1,2-diglyceride. The resulting phosphatidic acid diphenyl ester was then converted to phosphatidic acid by hydrogenolysis of the protecting phenyl groups (Fig. 9). The product had irreproducible melting points, probably because of the presence of partially neutralized forms of phosphatidic acid that are due to the alkali present in the Adams' catalyst, and/or because of the formation of lysophosphatidic acid. Both racemic sn-1.2 and sn-2,3-diacyl saturated phosphatidic acids were also prepared by this method (49). A method using 1,2-diacyl-sn-glycero 3-iodohydrin and silver dibenzylphosphate followed by hydrogenolysis of the protecting benzyl groups yielded saturated phosphatic acids (62, 51, 52).

The first synthesis of unsaturated phosphatidic acids was achieved by Baer and Buchnea (53) by phosphorylation of 1,2-dioleoyl-sn-glycerol with phosphorus oxychloride. Unsaturated derivatives of phosphatidic acid were also synthesized by van Deenen and de Haas (52). The procedure involved the protection of the double bonds of the mixed acid dibenzylphosphatidate (prepared from the 1,2-diacyl sn-glycerol 3-iodohydrin and the silver salt of dibenzylphosphate) by bromination at -20 °C, catalytic removal of the protecting benzyl groups and finally removal of the protecting bromines with activated zinc dust. A more recent synthesis of unsaturated and mixed acid phosphatidic acids (55) used di-t-butyl silver phosphate to react with 1,2-diacyl-sn-glycero-3-iodohydrin to yield di-t-butylphosphatidic acid. The protecting groups were removed under anhydrous and mild acid conditions that did not cause loss of the fatty acid side chains. Racemic diacylphosphatidic acids have also been prepared by Lapidot (56) by acylation of glycerophosphate with a fatty acid anhydride and the tetrammonium salt of the fatty acid with either saturated or unsaturated fatty acids. This is a direct and convenient procedure for many derivatives but suffers from the drawback that the phosphatidic acids formed are not optically active.

Phosphatidic acids can be conveniently prepared also from commercially available synthetic phosphatidyl cholines or from natural lecithins by the action of phospholipase D (Fig. 9) (57, 58, 59).

In the present work, saturated phosphatidic acids were synthesized both by a modified procedure of Baer (48) and by phospholipase D hydrolysis (59) of correponding PC's; unsaturated phosphatidic acids were made by the phospholipase D hydrolysis procedure (Fig. 9).

Figure 9: Two procedures used for the synthesis of *sn*-1,2-diacyl phosphatidic acids: A) method of Baer (74) for saturated PAs, B) enzymatic method for both saturated and unsaturated PAs

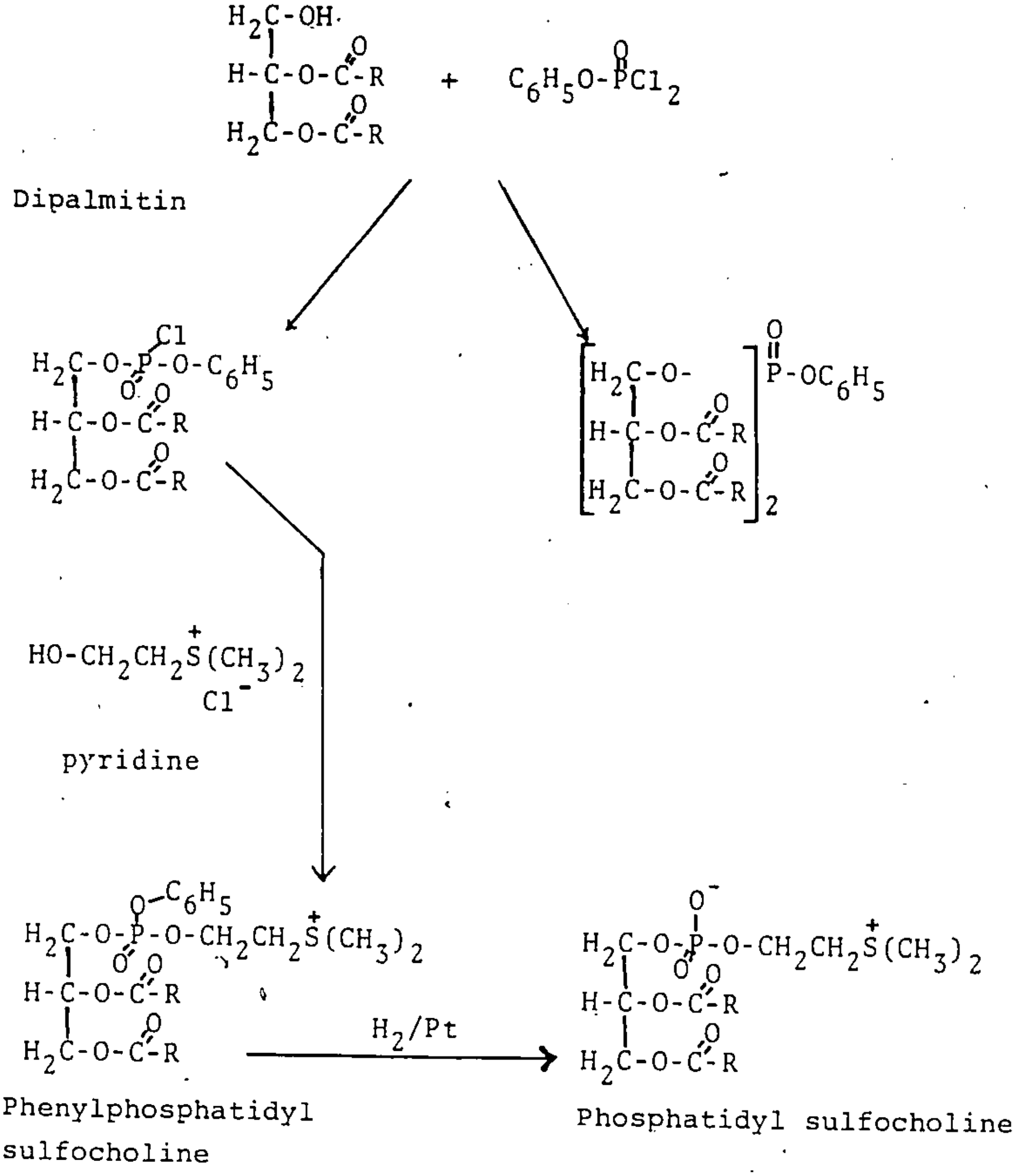

A
1,2-Diacyl-sn-glycerol
(C6H5O)2P(O)Cl
1,2-Diacyl-3-diphenyl-phosphate-sn-glycerol
PtO2 / H2
1,2-Diacylphosphatidic acid
Phospholipase D
B
A Phosphatidylcholine

1.1.5 Strategy

The synthesis of PSC was previously attempted in this laboratory using the method of Aneja (38). The labile nature of sulfocholine at high temperatures via β-elimination, especially in the presence of base, was probably the reason for the failure of this technique. When this thesis work was started, it was decided to attempt a method analogous to that of Eible et al (24). In the final step dimethyl sulfide was substituted for trimethylamine. Unfortunately, despite many attempts using various conditions, the substitution reaction failed to yield any PSC. We then decided to try another approach based on the well known acylation procedure of sn-glycero-3-phosphoryl choline to form PC (29), using glycerophosphosphorylsulfocholine (GPSC). However, this method proved not to be feasible because of the labile nature of GPSC in base. Therefore the classical method of Baer and Kates (20) was adapted for synthesis of PSC by substituting sulfocholine for choline. This method yielded the first synthetic DPPSC, albeit in low yield (Fig 10) and will be described in section 1.5.2.1.

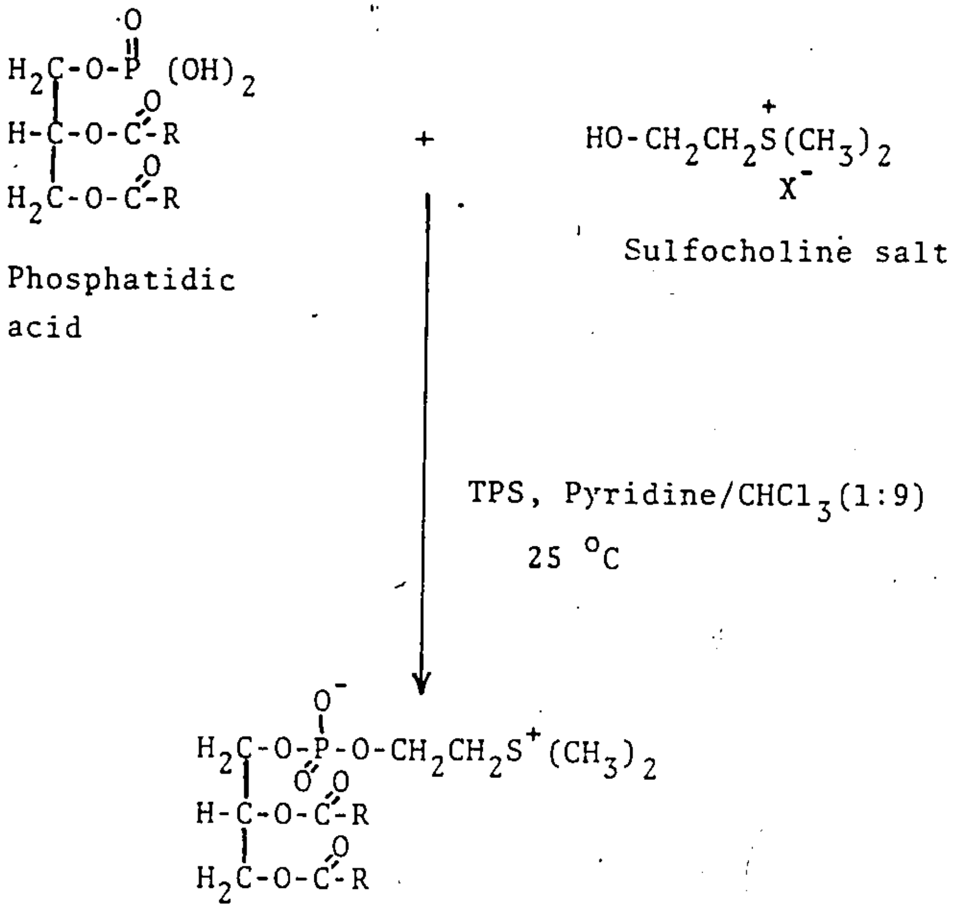
After this initial success, it was decided to synthesize PSC via a method that should give a higher yield and which would permit the synthesis of both saturated, unsaturated and deuterium labelled derivatives. The method of Aneja was modified to produce milder conditions by using a lower temperature and a lower concentration of pyridine base as described in section 1.5.3 (Fig. 9). The phosphatidic acids were prepared by enzymatic degradation of synthetic and natural PC's (Fig. 9). The synthesis of saturated phosphatidic acids was also carried out by the method of Baer (48). In addition the insoluble and hygroscopic chloride of sulfocholine was replaced by the soluble and stable non-hygroscopic tosylate in some syntheses and gave higher yields. The use of this derivative was suggested by Dr. Tony Durst, from the Chemistry Department of this University.

Figure 10: The chemical synthesis of saturated phosphatidyl sulfocholine by the method of Baer and Kates (20)


Dipalmitin
+
pyridine
H_2/Pt
Phenylphosphatidyl sulfocholine
Phosphatidyl sulfocholine

R = $CH_3(CH_2)_{14}$-

Figure 11: The chemical synthesis of phosphatidyl sulfocholine by the method of Aneja and Chadha (38).


Phosphatidic acid

Sulfocholine salt

TPS, Pyridine/$CHCl_3$(1:9)
25 °C

Phosphatidyl sulfocholine (Ix)

x

R=b $CH_3(CH_2)_{12}$-

a $CH_3(CH_2)_{14}$-

c $CH_3(CH_2)_{16}$-

d $CH_3(CH_2)_7\underset{H}{C}\ \underset{H}{C}(CH_2)_7$-

X = Cl, tosylate

1.2 MATERIALS

1.2.1 Solvents and reagents

All solvents were glass distilled, a 10% forerun being discarded. Anhydrous solvents were prepared essentially as described by Vogel (60) and/or Fieser and Fieser (61), with the following modifications:

1. Chloroform was washed with distilled water to remove ethanol, dried over anhydrous sodium sulfate, and distilled over phosphorus pentoxide (61.2 °C). It was discarded when traces of phosgene were detected by smell.
2. Diethyl ether and Tetrahydrofuran were refluxed with lithium aluminum hydride for one hour, and distilled carefully.
3. Pyridine was dried over KOH and barium oxide from which it was distilled. A constant boiling fraction (115.5 °C) was collected.
4. Carbon tetrachloride was purified and dried by distillation discarding the first 20 % of the distillate and collecting the constant boiling fraction at 76.8 °C. The water was removed in the forerun as a biphasic azeotrope boiling at 66.8 °C.

All purified solvents were stored in brown bottles over nitrogen. The solvents used for spectroscopic measurements were:

1. <u>Chloroform</u>: Fisher certified A.C.S. Spectranalysed (Fisher).
2. <u>Carbon tetrachloride</u>: Baker-Analysed GC Spectrophotometric quality solvent (Baker).
3. <u>Deuterochloroform</u>: "Silanor C", containing 1% (v/v) tetramethyl silane as reference standard (Merck, Sharp and Dohme, Canada).
4. <u>Hexadeuterated dimethylsulfoxide</u>: "Silanor" containing about 1 % (v/v) tetramethyl silane reference standard, (Merck, Sharpe and Dohme).
5. <u>Deuterium oxide</u>: (Merck, Sharpe and Dohme).
6. <u>Deuterium depleted water</u>: (Aldrich Chemical Company).

The following reagents were purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A.: triisopropylphenylsulfonylchloride, monophenyl phosphoryldichloridate.

The following reagents were prepared as follows:

1. <u>Ethereal solutions of diazomethane</u> were prepared according to Fieser and Fieser (p. 196, vol. 1) from p-toluenesulfonyl methylnitrosamide, on a one-quarter scale. It was stored at -20 °C as long as the yellow colour persisted.
2. <u>Palladium on carbon (10% catalyst)</u> was prepared according to Hessel et al. 1954 (62).

3. Platinum oxide or Adam's catalyst was obtained from Matheson Coleman and Bell, Norwood, Ohio, U.S.A. or prepared from chloroplatinic acid according to Vogel (60).

1.2.2 Enzymes

Phospholipase D: Phosphatidylcholine phosphatidohydrolase (E.C NO. 3.1.4.3) from cabbage was purchased from the Sigma Chemical Company, St. Louis Mo.

1.2.3 Microorganisms

1.2.3.1 Nitzschia alba

The nonphotosynthetic marine diatom, Nitzschia alba was the organism used for the isolation of the natural phosphatidyl sulfocholine and for metabolic studies (63). The organism was isolated, screened and studied by professor B.E. Volcani of the Scripps Institute of Oceanography at the University of California, San Diego, La Jolla, California 92093. The organism was grown in his laboratory and sent to Ottawa as a lyophilised off white powder.

1.3 METHODS

1.3.1 Chromatographic Methods

1.3.1.1 Column Chromatography

Both natural and synthetic lipid intermediates were purified on columns of Bio-Sil A (100-200 mesh) silicic acid. The silica was activated for 12 hours at 110 °C before use, cooled, immediately made into a slurry with chloroform and poured into a glass column equipped with a teflon stopcock above which a glass wool plug had been introduced. The adsorbant was allowed to settle by gravity while passing five column volumes of chloroform through the column. A solution of lipid mixture in a minimal volume of chloroform was introduced at the top of the column and the elution rate was adjusted according to the size of the column; for a fifty gram column 5 ml per minute was usually satisfactory.

Elution of the synthetic lipids was accomplished by passing a step gradient of chloroform and methanol solutions through the column. Typically ten column volumes of chloroform:methanol 20:1, 10:1, 9:1, 8:1, 6:1, 4:1, 2:1 and 1:1, were used and the PSC's were generally eluted in one of the last three solvents. At this stage the PSC's were chromatographically pure as judged by TLC. The fractions containing the PSC's were pooled and brought to dryness on a Buchi rotory evaporator. The PSC's were extracted by the Bligh and Dyer procedure (see section 1.3.2.1) and then precipitated with acetone to yield pure solid material. For the unsaturated derivatives the final acetone precipitation step was omitted.

Neutral alumina (70-230 mesh ASTM) (Brinkmann Instruments, Rexdale Ontario) was used to purify lecithin from hens' egg yolks. It was dried at 110 °C overnight and deactivated by addition of water up to 3 percent and mixing on a rotoevaporator before use. The elution was accomplished according to Hanahan (64).

1.3.1.2 Thin Layer Chromatography (TLC)

A Analytical TLC

For determination of purity and monitoring of reactions, thin layer chromatograms were run on 7.5 cm x 4.0 cm microscope slide plates or 20 cm x 20 cm plates coated with silica gel G (0.5 mm thickness). Spots were routinely visualised by spraying with one of the following reagents:

1. 50% sulfuric acid in ethanol followed by charring detects all organic material (65)
2. Dragendorff reagent for the detection of onium type compounds (e.g. Choline, Sulfocholine) (66, 67)
3. Phosphate detecting reagent of Dittmer and Lester 1964 (68) which gives blue spots in the presence of phosphorus containing compounds followed by charring

A combination of these sprays may be used on the same plate (e.g. the Dragendorff spray followed by the phosphorus spray). The Rf values quoted are the mean values of at least three independent determinations. Commercial plates were also used when better resolution was needed, such as the separation of PC and PSC. The plates were either Brinkman solil G-25 (0.25 mm thick layer) or Whatman Linear KS plates (0.25 mm thickness and 80 angstrom pore diameter).

B Preparative TLC

Preparative TLC was carried out on 20 cm x 20 cm plates coated with silica gel G (1 mm thickness). The coated plates were washed once by ascending chromatography in chloroform:methanol (1:1, v/v), air dried and then activated at 110 °C for 12 hours. Solvents systems used for the separation of neutral and polar lipids are given in the appropriate experimental sections.

For the separation of natural lipids or for the purification of PSC's, plates were streaked with a solution (80 mg/ml) of the lipids (ca. 40 mg/plate) in chloroform:methanol 95:5 v/v) using a 'Pelick Streaker' (Applied Science Laboratories, State College, Pennsylvania). The plates were developed in pre-equilibrated rectangular jars lined with Whatman no. 4 paper, using chloroform:methanol:water (65:35:5, by vol.) as solvent. The lipid bands were visualised with iodine vapour after air drying the plates, and the spots were marked. The iodine was then allowed to sublime away before scraping and eluting the lipids spots.

1.3.1.3 Paper Chromatography

The water-soluble products were identified by paper chromatography as follows:

5 microlitres aliquots containing about 40 micrograms of choline or sulfocholine were applied from a concentrating tube to a strip of Whatman no. 4 paper (19x57 cm). The choline derivatives were chromatographed for 12-16 hours (ascending technique) or 6-7 hours (descending technique) in one of the following solvent systems:

1. Butanol-propionic acid-water (5:2:3, by vol.), ascending,
2. Butanol-acetic acid-water (5:2:3, by vol.), descending,
3. Phenol-water (9:1, w/v), ascending.

The water-soluble components were visualized, after thorough drying of the paper in air, with the Dragendorff spray reagent to detect choline or with UV light (366 nm) to detect nucleotides. Radioactive compounds were located by autoradiography or by a Berthold Radioactive Scanner (Varian Aerograph series 6000.10).

1.3.2 Lipid Extraction Procedure

1.3.2.1 The Bligh and Dyer Procedure (69)

The Bligh and Dyer method was used to separate the lipid material from other classes of compounds (protein, carbohydrate, nucleic acids and salts etc.). It is a two step extraction procedure: The first step consists of disrupting cells in a one phase system containing methanol:chloroform:water in a ratio of 2:1:0.8, (by vol.). The lipids are soluble in this mixture while any insoluble material (certain proteins) is removed by filtration or by centrifugation. Chloroform and water is then added to achieve a final ratio of methanol:chloroform:water of 1:1:0.9, (by vol.). This is a two phase system with a lower chloroform layer containing the lipids and an upper methanol:water (10:9, v/v). There is usually a button of solid material at the interphase (protein). The lower chloroform layer is carefully removed, washed three or four times with an equal volume of upper phase (methanol:water, 10:9, v/v) and evaporated to dryness under vacuum in the presence benzene to eliminate water from the mixture.

Example extraction: to 2.5 g of diatom cells (dry weight) in a glass-stoppered 50 ml tube, 20 ml of methanol, 10 ml of chloroform, and 6 ml of water (assume 2 ml water in the cells) were added. The mixture was vortexed, centrifuged and the supernatant removed. The pellet was again extracted with 3.8 ml of methanol:choroform:water (2:1:0.8, by vol.) and the supernatant after centrifugation was combined with the first extract. 10 ml of chloroform and 10 ml of water were added and the mixture was centrifuged, to separate the two phases. The lower chloroform layer was removed carefully by Pasteur pipet (avoiding the protein layer at the interphase), washed thrice with 10 ml aliquots of upper phase (methanol:water, 10:9, v/v), and finally concentrated to a small volume on a rotary evaporator. Further purification was accomplished either by column chromatography or by preparative TLC.

With chromatographic eluates or with the residues from reaction mixtures a modification of the above procedure was used. This consisted in dissolving the lipid material in a mixture of methanol:chloroform (1:1, v/v, 1-10 mg/ml). The solution was cleared by centrifugation and 0.45 volumes of water was added to make two phases, in the same ratio as above. The mixture was treated as before. If iodine was present in the lipid sample (from TLC) 0.02 N sodium thiosulfate was substituted for the 0.45 volumes of water; similarly, for the elimination of pyridine from the reaction material or to convert certain lipids from the salt to the free acid form (e.g. calcium salt of phosphatidic acid to free phosphatidic acid) 0.2-2.0 N HCl was used depending on the concentration of lipid used.

1.3.2.2 Isopropanol Extraction (70, 65)

When enzymatic degradation of the lipids is likely to occur (e.g. by the action of lipases or phospholipases; especially in plants), the method of extraction used should result in inactivation of these enzymes. The Bligh and Dyer procedure does not completely inactivate hydrolytic enzymes but the use of hot isopropanol is very effective in deactivating enzymes as well as in extracting the lipids.

<u>Example extraction</u>: to 35 mg of lyophilized diatom cells was added 4 mL of hot isopropanol; the mixture was shaken and centrifuged, and the isopropanol extract was removed. The extraction with hot isopropanol was repeated 4 times followed by two additional extractions with 4 mL portions of chloroform:methanol (1:1, v/v). The extracts were pooled and the solvent was removed under vacuum in a rotary evaporator. The residue was immediately dissolved in 8 mL of chloroform:methanol (1:1, v/v), transferred to a 15 mL centrifuge tube, and diluted with 3.6 ml of water to form two phases. After centrifugation the upper methanol:water phase was carefully removed; the lower chloroform phase was washed three times with methanol:water (10:9, v/v) and finally concentrated under a stream of nitrogen to a known volume.

1.3.2.3 Acetone Precipitation (65)

Polar lipids, especially phosphatides, are insoluble in acetone (particularly in the presence of magnesium ion), while neutral lipids are soluble. This provides a convenient method for the separation of the two classes of lipids.

A chloroform solution containing lecithin or sulfolecithin cleared by centrifugation was concentrated to a small volume (taking care to assure that all the lipid remained in solution) and 20 volumes of ice cold acetone were added in portions with shaking. The white flocculent phospholipid precipitated out of solution and the tube was stoppered and stored for 12 hours at 4 °C for the saturated derivatives or at -20 °C for the unsaturated derivatives. The sample was centrifuged at 2000 x g for 5 minutes in the cold (4 °C); the pellet was resuspended in a small volume of ice cold acetone, recentrifuged and the final pellet was dried _in vacuo_ in a desiccator to remove traces of acetone.

1.3.3 Analytical Procedures

1.3.3.1 Dry Weight

An aliquot of the lipid solution containing 25 mg was placed in a 20 mL weighing bottle and the solvent was evaporated under a stream of nitrogen, taking care to spread the residue along the interior surface of the vessel. The residue was pumped under vacuum (0.1 mm Hg) to constant weight.

1.3.3.2 Phosphorus Determination

The total phosphorus content of the phospholipids was determined using a modification of the semimicro method of Allen (71), or the micro method of Bartlett (72). All glassware was washed with chromic acid prior to use.

A Allen's Method

An aliquot of sample solution containing 20-80 µg of P (less than 2 mg of lipid) was placed in "Lewis-Benedict sugar tube" and evaporated to dryness under a stream of nitrogen. The residue was digested for 4-5 minutes with 2.0 mL of 72 % perchloric acid (Analar, BDH Chemicals) at reflux temperature. To the cooled tubes, water was added to the 12.5 ml calibration mark and 2 mL of Amidol reagent solution * and 1 mL of ammonium molybdate solution ** were added with thorough mixing after each addition. After 20 minutes for colour development the solution was diluted to the 25 mL calibration mark and the tube was mixed by inversion. The absorbance was measured at 680 nm. Standard inorganic phosphate solutions were treated identically omiting the digestion step. Beer Lambert's law is obeyed in the range 10-100 ug of P. The following solutions were prepared for phosphate analysis:

1. * Amidol reagent 1.0 g of 2,4-diaminophenol dihydrochloride (Amidol) and 20 g of sodium bisulfite dissolved in 100 mL distilled water and filtered.

2. ** Ammonium molybdate solution: 8.3 g of ammonium molybdate dissolved in 100 mL of distilled water.
3. *** Standard Phosphate solution: 1.097 g of KH_2PO_4 (primary standard, Fisher Scientific Co.) dissolved in 250 mL of distilled water. This solution was diluted 10 fold to give a working solution containing 10 ug of P/mL for the Allen method and 100 fold to give 1 µg of P/mL for the Bartlett method.

B Modified Bartlett Method

Lipid samples containing 0.5 to 6 µg of P were digested in sugar tubes (25 mL, bulb shaped) with 0.4 mL of 72% perchloric acid. The tubes were cooled to room temperature and 4.2 mL of distilled water , 0.2 mL of amidol solution and 0.2 mL of ammonium molybdate solution were successively added with vortex mixing. The tubes were then covered with glass beakers and heated together with tubes containing standard amounts of phosphate (treated in the same way) in a boiling water bath for 7 minutes and cooled by immersion in a cold water bath. A stable blue colour was developed after 15 minutes and measured at 800 nm. Beer-Lambert's law was obeyed in the range of 0.5-10 µg of P.

1.3.3.3 Fatty Acid Determination

For the determination of fatty acid composition of ester-linked lipids and or quantitative determination of the fatty acids, lipids were methanolysed by the method described by Kates, 1972 (65). An aliquot of the chloroform solution of lipids (1-15 mg) and a known aliquot of a standard solution of heptadecanoic acid (0.5-7.0mg) was pipetted into a 15 mL straight-walled ground glass tube and the solvent evaporated under a stream of nitrogen; 4.5 mL of 3% HCl in methanol (made by dissolving 3.0 g of HCl gas in 100 mL of methanol) was added, the tube loosely stoppered, and heated in a dry heating block at 70 °C (Lab-line Instruments Inc., Temp Block Module Heater NO-2090) for one to two hours. After dilution with 0.5 mL of water, the mixture was extracted repeatedly with 1 mL aliquots of petroleum ether (b.p. 30-60 °C). The combined ether extracts were concentrated to a small volume (ca 5 μl) under a stream of nitrogen in a test tube with a fine tip. An aliquot containing about 100 ug of fatty acid methyl ester was analysed by gas-liquid chromatography as described below. The fatty acid composition was calculated from the peak areas as described by Carroll, 1961 (73) and expressed in area %. Absolute contents of fatty acids were determined with the aid of the internal standard methyl heptadecanoate.

1.3.3.4 Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) was carried out using a Carlo Erba Fractovap unit equipped with a flame ionization detector set at 240 °C and an injection port set at 240 °C.

Separation of fatty acid methyl esters was carried out on 10% SP2330 or 10% SP2300, both on 100/120 Chromosorb W AW (Sulpelco) packed in 1.8 mm x 0.4 (i.d.) glass column held at 175-200 °C with a nitrogen carrier gas pressure at 0.7 kg/cm^2. Sterol and sterol acetates were separated on 3% SE-30 on Chromosorb W packed in a 46 cm x 0.6 mm (i.d.) steel column held at 190 °C with a nitrogen gas pressure at 0.8 kg/cm^2.

1.3.4 Spectroscopic Analytical Methods

1.3.4.1 Infrared Spectoscopy

All pure compounds were analysed with a Pye Unicam double beam spectrophotometer (Model no SP1000). Spectra of solids were recorded as KBr pellets: 1 % solutions were found to be optimal for most polar lipids (molecular weight 600-800), while proportionally lower concentrations were used for smaller molecules. The lipid sample (2 mg) was added to finely ground KBR (190 mg), the mixture was ground further in an agate mortar for 5 minutes, then added to the KBr pellet maker (Barnes Engineering Company, Stampford, Connecticut, USA) and subjected to a vacuum of 1 mm of Hg for 10 minutes before applying a pressure of up to 400,000 psi of 5 minutes. The pressure was released, the pellet removed, and the spectrum measured immediately.

For small quantities of oil samples, spectra were recorded in KBr, as follows: a solution of the lipid (2 mg) in 0.2 mL of chloroform was added to 180 mg of finely ground KBr in a small centrifuge tube. The solvent was removed in vacuo. The mixture was ground and processed as described in the preceeding paragragh.

The spectra of oil, were also recorded as thin films between two NaCl plates when sufficient quantity of the material was available.

1.3.4.2 Nuclear Magnetic Resonance Spectrometry

Proton NMR spectra were measured using a Varian HA-100 NMR spectrometer (100 MHz) for critical spectra and on a Varian T-60 NMR spectrometer (60 MHz for routine measurement and purity checks. Samples were run routinely as 5% solutions in 4 mm (OD) thin walled tubes. For smaller samples thick-walled tubes with thick bottoms (0.5 cm) were used. Tetramethylsilane (TMS) was used throughout as an internal reference in $CDCl_3$ solutions and in hexadeuterated dimethyl sulfoxide solutions while no internal standard was used with heavy water. Whenever possible, chemical shifts were reported as δ (ppm) relative to TMS taken as zero ppm.

For identification of readily exchangable protons (e.g. OH) the sample was shaken with 2 drops of heavy water and the spectrum rerun. The signal of ionizable groups disappear in the presence of heavy water.

1.3.4.3 Mass Spectrometry

GC-mass spectrometry was used to analyse the structure of certain lipids of N. alba. Phospholipids were analysed by the technique called Field Desorption Mass-Spectrometry (FD-MS).

1.3.4.4 Optical Rotations

Optical rotations of samples in chloroform:methanol (1:1,v/v) solutions, at 25 °C, were measured at the sodium D line using a Perkin-Elmer polarometer, Model 141, with digital readout. Because of the weak optical activity of most of the substances investigated, readings were taken on sample solutions of at least 2% concentration in a 1 dm tube. Care was taken to ensure that traces of silica arising from prior chromatography were completely removed by centrifugation. The optical rotations reported are the mean values of at least five separate readings.

1.4 RADIOISOTOPIC PROCEDURES

1.4.1 Synthesis of ^{3}H, ^{14}C, and ^{35}S-Labelled Precursors

1.4.1.1 ^{3}H-Sulfocholine chloride

A mixture of 200 mg of (^{3}H)dimethylsulfate (5 mCi, specific activity 3.15 mCi/mmole), 300 mg (3.2 mmoles) of S-methyl-2- thioethanol and 1 mL of dry acetone was heated under reflux for 90 minutes and left to cool for 18 hours. The product separated from the solution as an oil, but crystallized when left in the cold at -20 °C for 36 hours. The crystals were washed with 8-10 mL of cold dry acetone and dried *in vacuo* to constant weight; the yield of crude product was 248 mg (100 %), containing 5 mCi, of ^{3}H (100% recovery of radioactivity). Paper chromatography using solvent system butanol:propionic acid:water (5:2:3, by vol.) showed the presence of the product together with an impurity; the ratio of radioactivity in the impurity relative to product was 1.8.

A solution of the crude product in 10 mL of water was passed through a column of 2.5 g of Rexyn (H^{+} form) cation exchange resin. The ^{3}H-sulfocholine was absorbed on the column while the impurity passed through. The product was then eluted with 2N HCl, and the eluates were lyophilysed and the residue made up to 10 mL in water; the total radioacitivy recovered was 3.0 X 10^{8} dpm (139 µCi) giving a specific radioactivity of 0.705 mCi/mmole. Paper chromatography in the same solvent system as above revealed a single Dragendorff-positive spot containing all the radioactivity.

1.4.1.2 ^{35}S-SULFOCHOLINE CHLORIDE

A mixture of 1.5 mCi (13.8 mCi/mmole) of ^{35}S mercaptoethanol, 46.5 mg non radioactive mercaptoethanol (total mercaptoethanol, 55 mg or 1.7 mCi/mmole) and 205 mg of methyl iodide was shaken in a tightly stoppered tube at room temperature for 60 hours. The dark oily product was purified on a 1.5 g Rexyn (H^+ form) cation exchange column, which was eluted with a step-wise gradient of HCl from 0.5 N to 6 N HCl. Sulfocholine appeared in the 1-2 N HCl fractions. The main impurity, trimethylsulfonium chloride, was eluted with 0.5 M, HCl while other polar bi-products of this reaction were eluted with 2 N HCl. The eluates were monitored by chromatography on Whatman No. 4 paper 1534 using the solvent system butanol:propionic acid:water (5:2:3, by vol.) and the appropriate fractions were lyophilized. The total radioactivity recovered was 4.3 X 10^8 dpm, giving a specific radioactivity of 1.16 mCi/mmole.

1.4.2 <u>Measurement of Radioactivity</u>

The radioactivity of ^{3}H, ^{14}C and ^{35}S-labelled compounds was measured by scintillation counting in plastic disposable vials in a scintillation cocktail consisting of 0.3 grams of POPOP, 5.0 g of PPO, 130 mL of methanol and 100 mL of solubilizer in one litre of toluene using a Beckman LS-150 scintillation counter. Each sample was counted to at least 1% statistical error and the ^{14}C and ^{35}S samples were corrected for quenching by the external standard ratio method using a series of standards containing equal amounts of ^{14}C toluene (0.03 x 10^5 dpm) but different amounts of the quenching agent chloroform. Radioactive spots on TLC plates were scraped directly into the above scintillation cocktail and counted. Radioactive spots on paper chromatograms were cut out and added directly to the scintillation mixture and their radioactivity counted.

1.4.3 Autoradiography

^{14}C and ^{35}S-labelled lipid spots on TLC plates and paper chromatograms were detected by exposing the chromatograms to a sheet of Kodak Blue Brand X-ray film for a suitable period of time depending on the amount of ^{14}C and ^{35}S radioactivity of the spots (2 days per 1000 dpm for TLC plates and 20 days per 1000 dpm for paper chromatograms). The film was developed and fixed by standard photographic techniques. Autoradiograms of ^{3}H-labelled compounds required the use of a sensitizer. This is a phosphorescent product from New England Nuclear which is sprayed on the plate or paper before exposing to X-ray film at -80 °C for 72 hours per 1000 dpm.

1.5 EXPERIMENTAL

1.5.1 Synthesis of Precursors to PSC

1.5.1.1 Preparation of S-methyl-2-thioethanol

To a solution of 2-mercaptoethanol (15.6 g , 0.2 moles) in 5 M methanolic KOH (40 mL) was added dropwise, over a period of one hour, a solution of methyl iodide (28.4 grams, 0.2 moles) in 15 mL of methanol at room temperature. The mixture was stirred for sixteen hours and then filtered to remove potassium iodide. The filtrate was diluted with an equal volume of water and extracted with several 50 mL portions of methylene chloride. The combined extracts were dried over anhydrous sodium sulfate and concentrated to dryness under reduced pressure at room temperature. The residual 2-(methylthio)-ethanol was distilled under reduced pressure; the boiling point was 60-62 °C at 10 mm of Hg and the yield was 12.0 g (66 %). The NMR spectrum obtained was consistent with the expected structure and showed the following signals (δ ppm): 2.08 (singlet), S-CH_3 (3H); 2.70 (multiplet), CH_2S (2H); 2.74 (singlet), OH (1H); 3.75 (multiplet), CH_2O (2H).

1.5.1.2 Preparation of S,S-Dimethyl-2-hydroxyethylsulfonium Iodide

The sulfonium iodide was prepared in quantitative yield by reacting 2-(methylthio)-ethanol with a five-fold excess of methyl iodide at room temperature for 16 hours. After removal of the unreacted methyl iodide by pipet the product formed a solid mass which was freed of final traces of solvent *in vacuo*. The residue was then dissolved in a minimal amount of methanol, precipitated by the addition of 20 volumes of dry acetone, filtered and finally washed with cold anhydrous acetone and dried *in vacuo*. The chromatographically pure sulfocholine iodide [Rf 0.7 in the solvent system butanol:propionic acid:water (5:2:3, by vol.) and 0.9 in the solvent system phenol:water (9:1, w:v)] was obtained in an overall yield of 63%. The NMR spectrum showed signals at the following values in δ (ppm): 3.01 (singlet), $S(CH_3)_2$ (6H); 3.6 (multiplet), CH_2O (2H); 4.6 (multiplet), OH (1H).

1.5.1.3 Preparation of S,S-Dimethyl-2-hydroxyethylsulfonium Chloride

The sulfonium chloride was prepared by passage of an aqueous solution of the iodide through a column of Rexyn H^+ (101) cation exchange resin, followed by elution with 2N HCl, removal of the water and HCl by lyophilization *in vacuo* over KOH and crystallization of the sulfonium chloride from acetone. This compound is very hygroscopic and was dried in a pistol over phosphorus pentoxide at 40 °C at 1 mm Hg before use. It was quickly ground to a fine powder and dried again before use. The proton NMR spectrum (Fig. 12) in D_6-dimethylsulfoxide showed the same signals as sulfocholine iodide.

1.5.1.4 Preparation of S,S,-Dimethyl-2-hydroxyethylsulfonium Tosylate

The sulfonium tosylate was prepared by the reaction of 2-(methylthio)-ethanol (1 mole) with methyl-p-toluenesulfonate (1.05 mole) in anhydrous acetone for 16 hours under reflux. After removal of the solvent <u>in vacuo</u> the residual oil was crystallized from dry ethyl ether. The yield was 86%. The NMR spectra in hexadeuterated dimethylsulfoxide was consistent with the structure.

1.5.1.5 Preparation of S,S-Dimethyl-2-hydroxyethylsulfonium Sulfate

The sulfonium sulfate was prepared by the reaction of 2-(methylthio)- ethanol (2 moles) with dimethylsulfate (1 mole) in anhydrous acetone for 2 hours under reflux. After removal of the solvent <u>in vacuo</u> the residual oil failed to crystallize from cold dry ethyl ether or from dry acetone. The sulfate assumes a semi-solid white appearance when dried over phosphorus pentoxide. It is soluble in water but insoluble in methanol, ether, or acetone.

1.5.1.6 Preparation of Diglycerides

The synthesis of diglycerides is central to many of the <u>de novo</u> syntheses of lecithin and other phospholipids. To obtain optically active diglyceride e.g 1,2-diacyl <u>sn</u>-glycerols a lengthy synthetic route starting from D-mannitol is generally used (Fig. 13). Following the method of Baer (74), acetone was reacted with D-mannitol to obtain 1,2,5,6-diisopropylidene-D-mannitol, which, after periodate cleavage and sodium borohydride reduction according to Lecocq and Ballou (75), yielded 1,2-isopropylidene sn-glycerol (with a specific rotation at the sodium D line of +15.3). The free alcohol was then benzylated according to Kates (76) and the isopropylidene group was removed with acid to obtain the key starting material for the synthesis of all the saturated diglycerides: 3-benzyl-sn-glycerol. Following the method of Sowden and Fisher (77) benzylglycerol was then acylated with fatty acyl chlorides in carbon tetrachloride to yield 1,2-diacyl-3-benzyl-sn-glycerol which upon hydrogenolysis with Pd as catalyst gave the saturated optically pure diglyceride.

Of course only saturated diglycerides can be obtained by this method. Unsaturated diglycerides are most conveniently prepared by hydrolysis of the corresponding lecithin with phospholipase C. The unsaturated lecithins can be synthesized from sn-3-glycerophosphoryl choline and an activated unsaturated fatty acid by a method such as that of Warner and Benson (36).

Figure 12: The proton NMR of sulfocholine chloride in hexadeuterated dimethylsulfoxide.

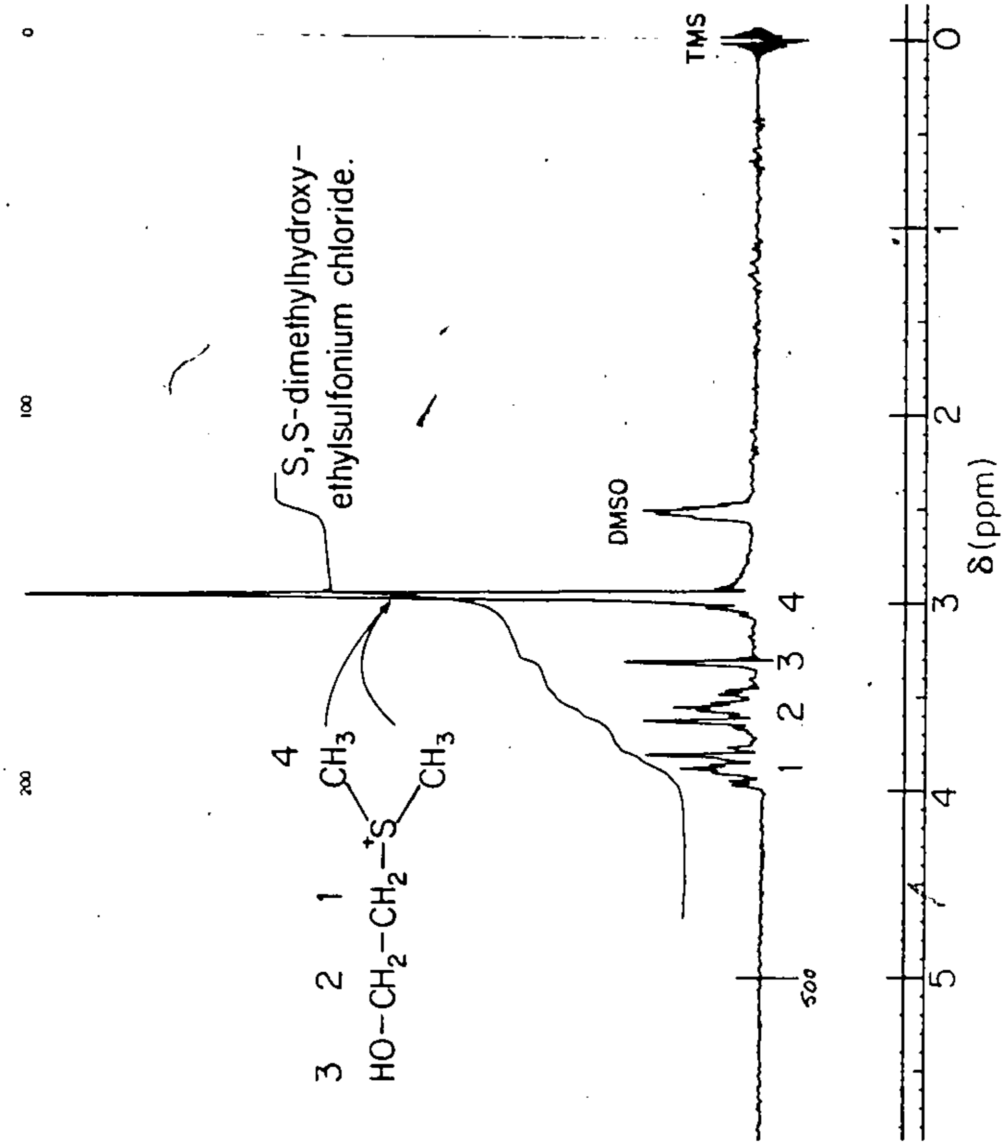
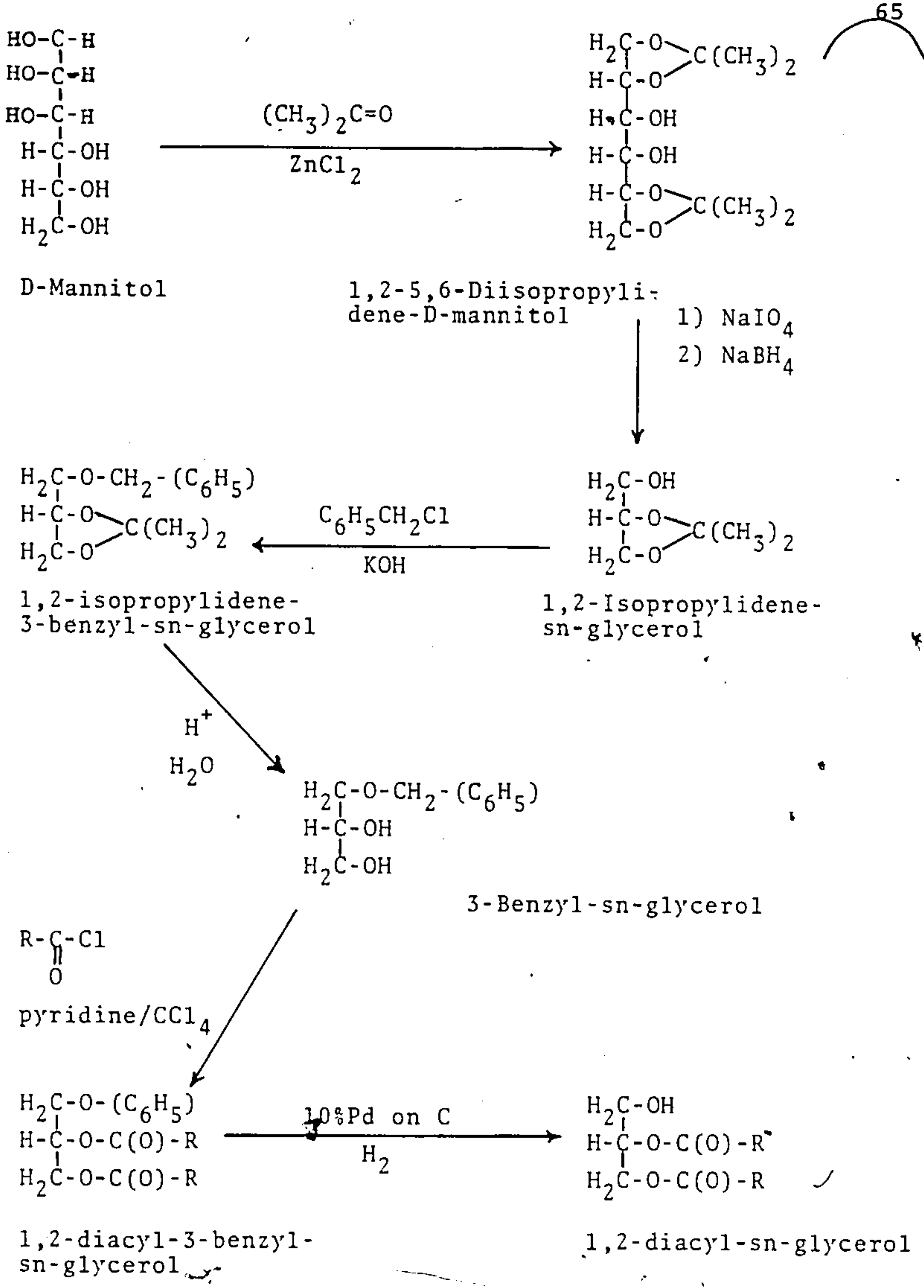

S,S-dimethylhydroxy-
ethylsulfonium chloride.
TMS
DMSO
3 2 1
HO–CH2–CH2–S+
4 CH3
CH3
1 2 3 4
0
100
200
500
0 1 2 3 4 5
δ(ppm)

Figure 13: The chemical synthesis of saturated diglycerides starting with D-mannitol by the procedure of Sowden and Fisher (77)



D-Mannitol

1,2-5,6-Diisopropylidene-D-mannitol

1,2-Isopropylidene-sn-glycerol

1,2-isopropylidene-3-benzyl-sn-glycerol

3-Benzyl-sn-glycerol

1,2-diacyl-3-benzyl-sn-glycerol

1,2-diacyl-sn-glycerol

1.5.1.7 Preparation of Phosphatidic Acid from Diglycerides

Optically active saturated phosphatidic acids were synthesized from 1,2 diacyl-sn-glycerol by the method of Baer (48). Thus, synthetic di-14:0 DG, di-16:0 DG and di-18:0 DG were phosphorylated with diphenylchlorophosphate in pyridine. The intermediate 1,2-diacyl diphenyl phosphates were converted to the corresponding phosphatidic acids by hydrogenolysis with platimum oxide catalyst. The optically active PA's were converted to the free acid form by the addition of 0.45 volumes of 0.2 N HCl to a 10% solution of chloroform:methanol (1:1, v/v). The lower chloroform phase was removed and evaporated to dryness under reduced pressure in the presence of benzene. The free phosphatidic acids then were used immediately because they are unstable and spontaneously deacylate to form lyso PA.

The phosphatidic acids were pure as judged by TLC in the solvent systems chloroform:methanol:ammonia (65:35:5, by vol.) and chloroform:methanol:acetic acid (65:35:5, by vol.). The infrared and NMR spectra of the free PA's and their phenyl derivatives gave the expected signals. The yields of the various saturated phosphatidic acids from DG were always above 80%.

1.5.1.8 Preparation of Phosphatidic Acid by Phospholipase D Method

Dioleoylphosphatidic acid, dipalmitoylphosphatidic acid and the phosphatidic acid from egg phosphatidyl choline were made by the enzymatic hydrolysis of the corresponding phosphatidyl cholines, with phospholipase D, by a modification of the method described previously (57) as follows (Fig. 9): to a suspension of 1 mmole of the vacuum dried lecithin (786 mg) in 15 mL of 0.2 M acetate buffer (pH 5.6) was added 3 mL of 1.0 M calcium chloride, 12 mL of enzyme solution (48 mg of Phospholipase D in 12 ml of water; specific activity 18 units/mg; Sigma Corp.) and 15 ml of ethyl ether (washed with water to remove traces of alcohols). The mixture was shaken vigorously at room temperature for 18 hours on a Burrell wrist action shaker. The ether was removed on a rotary evaporator and the aqueous suspension was diluted with 82 ml of methanol, 41 mL of chloroform and 3 mL of 2N perchloric acid, mixed, and diluted further with 41 mL of chloroform and 41 mL of water. The two phase mixture was allowed to separate in a separatory funnel and the lower chloroform phase was removed, diluted with benzene and brought down to dryness on a rotary evaporator. The dried residual oil was dissolved in 10 mL of chloroform and placed on a column of 20 g of silica gel (Bio Sil A, made up in chloroform), the free acid phosphatidic acid was eluted with 10 column volumes of chloroform:methanol (4:1, v/v). The solvent was removed in vacuo and the residual free phosphatidic acid was dried in vacuo over phosphorus pentoxide. The yield of chromatographically pure product was 90%.

1.5.1.9 Synthesis of Dioleoyl PC

Dioleoyl phosphatidyl choline was synthesized by the method of Warner and Benson (36) with a few modifications as follows: the activated oleoylimidazole was prepared by the reaction of 5 g (17.7 mmoles) of oleic acid and 3.3 g (18.0 mmoles) of 1,1-carbonyldiimidazole in 17 mL of THF. To the evaporated THF solution (oleoylimidazole) was added 15 mL of dimethylsulfoxide, 2.47 g (4.4 mmoles) of L-α-glycerophosphocholine cadmium complex in 15 mL of dimethylsulfoxide and 60 mL of sodium dimethylsulfoxide carbanion (0.9 grams, 36.5 mmoles) of sodium in 70 mL of dimethylsulfoxide. After 10 minutes stirring, 550 mL of 0.5 M HCl was added, then 3 portions of 435 ml of chloroform:methanol (2:1, v/v) were added with the lower chloroform layers being removed after each wash and pooled. The 600 mL of chloroform was extracted with 6 portions of 150 mL of neutral upper phase (methanol:water, 10:9, v/v) and was concentrated under vacuum to 7.70 g of oily residue. This was placed on a 110 gram silica gel column and was eluted with a step gradient of chloroform:methanol starting with pure chloroform and ending with chloroform:methanol (1:1, v/v). Fractions containing single phosphate positive and Dragendorff positive spots as visualized by TLC were pooled and used directly for the synthesis of dioleoylphosphatidic acid by enzymatic hydrolysis with phospholipase D (57) (see preceeding section)

1.5.2 Synthesis of Phosphatidyl sulfocholine

1.5.2.1 The Method of Baer and Kates (20)

This was the first synthetic method used for the synthesis of PSC. Though the yield was low, it provided a very pure product after one column chromatography step.

1,2-Dipalmitoyl-sn-glycerol (2.56 grams; 4.5 mmoles; $[\alpha]_D$ -2.6 degrees) was phosphorylated in a mixture of chloroform (25 mL) and anhydrous pyridine (1.5 mL, 5 mmoles) with monophenyl phosphoryl dichloride (1.05 grams, 5 mmoles) as described previously by Baer and Kates 1950 (20), followed by further addition of anhydrous pyridine (2 mL) and reaction with sulfocholine chloride (0.75 grams, 5.2 mmoles) at room temperature with stirring for 2 days.

After addition of 2 mL of water and stirring for 15 minutes to hydrolyse any unreacted phenylphosphoryl chloride, the mixture was partitioned according to a modified Bligh and Dyer procedure (69) by adding 25 mL of methanol and 20 mL of 2 N HCl. After mixing and centrifugation, the upper methanol:water layer was removed and the lower chloroform layer was washed 3-4 times with 5 mL portions of neutral upper phase (methanol:water, 10:9, v/v). The neutral chloroform extract was concentrated under reduced pressure using benzene to aid in removing traces of water. The residue (2.18 grams) was fractionated on a column of Bio Syl A silicic acid (20 grams) using chloroform (500 mL) to elute the fast moving components (dipalmitoylglycerol, bis (dipalmitoylglycerol)phenylphosphate) (Fig. 10) followed by 150 mL of chloroform:methanol (1:1, v/v) to elute the product phenyl phosphatidyl sulfocholine (phenylPSC), together with some monophenylphosphatidic acid (derived from chloromonophenylphosphatidate). PhenylPSC was then obtained in pure form for analytical purposes by preparative thin layer chromatography on silica gel H plates using the solvent system chloroform:methanol:acetic acid (90:10:1, by vol.). It gave a single spot with an Rf of 0.21 and positive phosphate and positive Dragendorff reactions.

The NMR spectra showed the expected signals in δ (ppm): 0.90 (triplet), CH_3; 1.3 (singlet), CH_2 ; 2.3 (triplet) CH_2CO; 3.4 (singlet), $S(CH_3)_2$; 4.1 (triplet), CH_2S; 4.3, (triplet of triplet), CH_2-OP (sulfocholine); 4.3, CH_2OP (glycerol); 5.3, CH-OC (glycerol); 8.1 (triplet), 8.5 (triplet) and 8.98 (triplet), phenyl-hydrogens.

The fraction (2.1 grams) eluted with chloroform:methanol (1:1, v/v) containing compound phenylPSC (Fig. 10) was hydrogenated in 50 mL of methanol (warmed to ensure dissolution) with 0.8 g of platinum oxide (PtO_2) under a slight positive pressure of hydrogen at room temperature. Hydrogenation was complete after one hour (uptake 360 mL at NTP, calculated for the cleavage of phenyl group and reduction of catalyst = 364 mL). The catalyst was removed by centrifugation and washed four times with 5.0 mL portions of chloroform:methanol (1:3, v/v). The combined supernatants were concentrated to dryness under reduced pressure and the residue was fractionated on a silicic acid column (80 grams, Bio Syl A) using 2.0 L of chloroform:methanol (4:1, v/v) to elute any fast moving components (phosphatidic acid and bis-PA) followed by 2.0 L of chloroform:methanol (1:1, v/v) to elute the desired compound 1,2-dipalmitoyl-sn 3-phosphatidyl sulfocholine. The combined eluates containing 16:0-PSC were brought to dryness on a rotary evaporator and the residue (380 mg, 11% yield from dipalmitin) was dissolved in 30 mL of chloroform:methanol (1:1, v/v) and diluted with 13.5 mL of distilled water. The biphasic mixture was centrifuged and the lower chloroform was washed 3-4 times with 5 ml portions of neutral upper phase (methanol:water, 10:9, v/v) and brought to dryness on the rotary evaporator. The residue was precipitated from chloroform solution (2 mL) by addition of anhydrous acetone (40 mL); the white flocculant product Ia was recovered by centrifugation, washed with cold acetone and dried in vacuo giving a yield of 325 mg (11 %) of chromatographically and analytically pure phosphatidyl sulfocholine.

Analysis: Calcd. for $C_{39}H_{77}O_8PS.H_2O$ (755.07):

C, 62.04; H, 10.41; P, 4.10; S, 4.25.

Found C, 61.96; H, 10.25; P, 4.23; S, 4.09.

$[\alpha]_D$= +7.56 ° (c, 5.25 in chloroform). M_D = +57.1 °.

1.5.3 Method of Aneja

1.5.3.1 Dipalmitoyl-sn-3-phosphatidyl sulfocholine (Ia)

A mixture of 1.14 g (1.76 mmoles) of 1,2-dipalmitoyl-sn-glycerophosphoric acid (Fig. 11) and 3.0 g (21 mmoles) of sulfocholine chloride was dried *in vacuo* (1 mm Hg) over phosphorus pentoxide overnight; 50 mL of anhydrous ethanol-free chloroform, 5 mL of anhydrous pyridine and 1.6 g (5.3 mmoles) of triisopropylbenzenesulfonyl chloride (TPS) were then added and the mixture was stirred vigorously overnight (20 hours at room temperature). The mixture was diluted with benzene, concentrated on a rotary evaporator at 35 °C and then dried in a desiccator over concentrated sulfuric acid *in vacuo* (10 mmm Hg) to remove pyridine. The yellow residue was dissolved in 30 mL of chloroform:methanol (1:1, v/v), diluted with 13.5 mL of water and the mixture was centrifuged. The lower chloroform phase was washed twice with 10 mL of acid upper phase (methanol:0.5 N HCl, 10:9, v/v) to remove traces of pyridine and sulfocholine and finally extracted twice with neutral upper phase (chloroform:methanol, 10:9, v/v) or until the washing was neutral. After concentration of the chloroform solution to dryness *in vacuo*, the white residue (2.1 grams) was fractionated on a silicic acid column (80 grams) by elution with 30 column volumes of chloroform:methanol (4:1, v/v) to remove the fast moving material, and then with 30 column volumes of chloroform:methanol (1:1, v/v) to remove the desired product Ia; lyso compounds were subsequently eluted with 10 column volumes of chloroform:methanol (1:2, v/v). The eluates containing compound Ia were concentrated to dryness _in vacuo_, and the residue was precipitated from a minimum volume of a solution made up in chloroform:methanol (1;1, v/v) by the addition of 20 volumes of cold acetone as described above; the yield of chromatographically pure compound Ia was 0.61 g (0.83 mmoles; 47 % from phosphatidic acid.) For analytical purposes, the compound was further purified by preparative TLC in solvent system chloroform:methanol:water (65:36:5, by vol.). The chloride of sulfocholine is very hygroscopic and is insoluble in the reaction mixture. On the other hand the tosylate is much less hygroscopic; it can be heated under vacuum in a pistol at 50 °C and it is soluble in the reaction mixture.

analysis: cald. for $C_{39}H_{77}O_8PS.H_2O$ (755.07 mw)

C, 62.04; H, 10.41; P, 4.10; S, 4.25.

Palmitic acid/P mole ratio, 2.00.

Found C, 62.08; H, 10.39; P, 4.15; S, 4.42.

Palmitic acid/P, 1.99.

$[\alpha]_D$ = +7.44 °, c: 1.15 in chloroform-methanol 1.1.

M_D : +56.2°.

Transition temperature: 81-83 °C
(Fig. 16).

1.5.3.2 Dimyristoyl-*sn*-3-phosphatidylsulfocholine Ib

Compound Ib (Fig. 11) was synthesized as described above for compound Ia by the method of Aneja, starting with 1.6 g (2.7 mmoles) of 1,2-dimyristoyl-*sn*-3-glycerophosphoric acid, 1.74 g (12 mmoles) of sulfocholine chloride and 3.29 g (9.6 mmoles) of TPS in 80 mL of chloroform and 10 mL of pyridine. The product was isolated and purified by silicic acid column chromatography, as described for Ia. The yield of pure product lb was 0.95 g (1.3 mmoles, 50 %).

Analysis: Calcd. for $C_{35}H_{69}O_8PS.H_2O$ (689.96 molecular weight),

C, 60.15; H, 10.09; P, 4.43; S, 4.59;
Myristic acid/P mole ratio, 2.00

Found C, 60.11; H, 10.10; P, 4.50; S, 4.63;
myristic acid/P mole ratio, 1.98.

$[\alpha]_D$ = +8.28 °, c: 1.15 in chloroform-methanol 1:1,v/v.
M_D: +57.9 °
Transition temperature by DSC, 55-58 °C.

1.5.3.3 Distearoyl-sn-3-phosphatidyl sulfocholine (Ic)

Compound Ic was synthesized as described above for compound Ia, starting with 1.5 g (2.1 mmoles) of 1,2-distearoyl-sn-3- glycerophosphoric acid (Fig. 11), 2.5 g (9 mmoles) of sulfocholine tosylate and 3.2 g (9.6 mmoles) of TPS in 50 mL of chloroform and 7 mL of pyridine. The yield of pure product Ic was 1.1 g (1.2 mmoles, 56 %).

Analysis Calcd. for $C_{43}H_{85}O_8PS.H_2O$ (molecular weight 811.10)

C, 63.67; H, 10.81; P, 3.82; S, 3.95;
Stearic acid/P mole ratio, 2.00

Found C, 63.58; H, 10.64; P, 3.95; S, 3.90;
Stearic acid/P mole ratio, 2.01.

$[\alpha]_D$ = +6.94 °, c: 2.52 in chloroform-methanol, 1:1, v/v.
M_D: +56.3 °
Transition temperature by DSC, 87-90 °C.

1.5.3.4 Dioleoyl-sn-3-Phosphatidyl sulfocholine (Id)

Compound Id was synthesized as described above for compound Ia, starting with 0.9 g (1.28 mmoles) of 1,2-dioleoyl-sn-3- glycerophosphoric acid, 2.3 g (5.4 mmoles) of sulfocholine tosylate and 1.9 g (6.3 mmoles) of TPS in 45 mL of chloroform and 6 mL of pyridine. The yield of TLC pure product ld after column chromatography was 0.74 g (0.92 mmoles, 72%); acetone precipitation was avoided because of losses due to the appreciable solubility of Id in acetone.

Analysis: Calcd. for $C_{43}H_{81}O_8PS.H_2O$ (807.16 molecular weight),
C, 63.99; H, 10.24; P, 3.84; S, 3.97.
oleic acid/P mole ratio, 2.00.

Found C, 63.32; H, 10.26; P, 3.91; S, 3.89.
oleic acid/P mole ratio, 2.00.

$[\alpha]_D$ = +7.0 °, c: 1.54 in chloroform-methanol 1:1. v/v.
M_D: +56.5 °.

1.5.3.5 Phosphatidyl sulfocholine analogue of Egg Yolk PC

This analogue was prepared as described above for Id, starting with 0.90 g (11.29 mmoles) of phosphatidic acid derived from egg yolk phosphatidyl choline (see above), 2.3 g (5.4 mmoles) of sulfocholine tosylate and 1.9 g (6.3 mmoles) of TPS in 45 mL of chloroform and 6 mL of pyridine. The yield of pure product was 0.7 grams (70 %).

Analysis Calcd. for $C_{41.2}H_{81.6}O_8PS.H_2O$ (molecular weight=786.1)
C, 62.95; H, 10.46; P, 3.94; S, 4.07.
Fatty acid/P mole ratio, 2.00.

Found C, 62.79; H, 10.41; P, 3.96; S, 3.93.
Fatty acid/ P mole ratio, 1.99.

Fatty acid composition: 16:0, 43.1%; 16:1, 0.9%; 18.0, 12.7 ; 18:1, 37.9%; 18.2, 1.8%; 20:1, 0.8%; >20:x, 2.8%.

1.5.3.6 Synthesis of 1,2-Dipalmitoyl-d4-*sn*-glycero-3-Phosphosulfocholine

To the cadmium chloride complex of glycerophosphocholine (200 mg, 0.48 mmole) rendered anhydrous by repeated evaporation of added dry benzene was added 12 mL of freshly distilled anhydrous alcohol-free chloroform. To this suspension palmitoyl anhydride-d4 (deuterated in the C2 methylenes) (690 mg, 1.2 mmoles) and 4-N,N-dimethylamino pyridine (120 mg, 0.96 mmoles) were added and the flask was sealed under dry nitrogen. The reaction mixture was stirred in the dark for 18 hours. The progress of the reaction was monitored by TLC using the solvent system chloroform:methanol:ammonia (65:35:5, by vol.). The mixture was diluted with 12 mL of methanol followed by 10.8 mL of 2N HCl and the lower phase was removed and quickly washed with methanol:water, (10:9, v/v). The chloroform phase was evaporated to dryness on a rotory evaporator. The 4-N,N-dimethylamino pyridine was removed by passing the mixture through a Rexyn column (102, H+). The crude lipid mixture was purified by preparative TLC using the solvent system chloroform:methanol:water (65:35:5, by vol.); the yield of pure d-4 DPPC was 222 mg (62%). The corresponding d-4 dipalmitoyl phosphatidic acid was prepared by the phospholipase D method, and then the sulfocholine head group was added by the Aneja method desribed in section 1.5.3.1, using sulfocholine tosylate instead of the chloride. The yield of pure d-4 PSC was 80 mg (36 %).

1.6 RESULTS AND DISCUSSION

1.6.1 Synthesis of Phosphatidyl sulfocholine

The synthesis of PSC was accomplished by two synthetic procedures, one based on the method of Baer and Kates (20), and the other on the method reported by Aneja and Chadha (38).

1.6.1.1 The Method of Baer and Kates

This synthesis (Fig. 10) involved the phosphorylation of an equivalent of a diglyceride (e.g. 1,2 dipalmitoyl-sn-glycerol) with an equivalent of monophenyl phosphoryl dichloridate. Unfortunately a second diglyceride can react with the intermediate product and give rise to the formation of bis-(dipalmitoyl glycerol) phenylphosphate, which lowers the yield of the overall reaction. Reaction with sulfocholine in a ten fold excess of pyridine then yields 1,2-dipalmitoyl sn-glycero phenylphosphoryl sulfocholine, or phenyl derivative of PSC.

This step has three difficulties, the extremely hygroscopic nature of sulfocholine chloride, its thermolability, and its insolubility in the reaction mixture. The first two difficulties were overcome by drying the sulfocholine in a pistol at room temperature and then quickly adding it to the reaction mixture. All these difficulties could have been avoided by the use of sulfocholine tosylate instead of the chloride, as was done in later syntheses using the Aneja method.

The phenyl PSC intermediate was partially purified using the Bligh and Dyer procedure followed by column chromatography. A small sample was purified by preparative TLC for analytical purposes. The semi-purified phenyl-PSC was subjected to catalytic hydrogenolysis to remove the protecting phenyl group and the desired phosphatidyl sulfocholine was purified by the Bligh and Dyer method and then by column chromatography on silicic acid. The overall yield of PSC, based on dipalmitin was low: 11 % comparable to the reported yields for phosphatidyl choline.

The hydrogenolysis step also involved certain problems. The presence of sulfur in the substrate seemed to poison the platinum catalyst, but this difficulty could be overcome by a second addition of the catalyst. Also, in one preparation, hydrogenation of the phenyl group occurred without cleavage. Finally the hydrogenolysis step limits this synthesis to saturated derivatives. In summary, this procedure would not be the method of choice, although it does readily yield the desired compound in a pure form.

1.6.1.2 The method of Aneja

The second method (Fig. 11), a modification of the method of Aneja (38), involved the coupling of a phosphatidic acid (6 derivatives in this case) with sulfocholine in the presence of triisopropylbenzenesulfonyl chloride, pyridine and anhydrous chloroform. The principal differences between this method and that of Aneja is the presence of chloroform and the lower temperature of the reaction (25 °C). The soluble sulfocholine tosylate was used preferentially. The product was purified by neutral and acid Bligh and Dyer (to remove traces of pyridine). The crude mixture was then purified by column chromatography on silicic acid. For analytical purposes and for the physical studies the product was further purified by preparative TLC. Minor amounts of impurities, probably traces of TPS, TPS sulfocholine ester, and sulfocholine, were coeluted with PSC from the column. These were detected by mass spectroscopy.

In principle, any lecithin could be synthesized by Aneja's method, saturated, unsaturated or mixed fatty acid lecithins. In the present study, 14:0 PSC, 16:0 PSC, 18:0 PSC, 18:1 PSC, the PSC derived from egg PC and two deuterated derivatives of PSC, were prepared by this method. Yield of the PSCs before TLC was 50 to 72 %. TLC purification resulted in the loss of about 20 % of the product by weight. PSC decomposes slowly even on storage at -80°C with the formation of lyso PSC and a faster moving component containing phosphorus but no sulfonium group (probably a phosphatidyl derivative). These degradative products were not investigated further.

1.6.2 Characterization of PSC

The purified acetone precipitated saturated derivatives of PSC were white amorphous solids at room temperature, while the purified unsaturated derivatives of PSC were waxes at room temperature. All the PSC's were chromatographically pure (Fig. 14) and co-chromatographed with the PSCs derived from *N. alba*. The elemental analyses gave good agreement between the calculated values for monohydrated PSC's and the experimental values. The synthetic analogues all had S/P atomic ratios close to the expected atomic ratio of 1:1 (Table 1) and the fatty acid/P mole ratios were very close to the theoretical value of 2:1 (Table 1).

TABLE 1

Mole Ratio of S/P and Fatty acid/P for PSCs

		MOLE RATIO			
		FOUND		CALCULATED	
Method	PSC	S/P	FA/P	S/P	FA/P
Aneja & Chadha (38)					
	di-14:0	1.01	1.98	1.00	2.00
	di-16:0	1.03	1.99	1.00	2.00
	di-18:0	0.95	2.01	1.00	2.00
	di-18:1	0.96	2.00	1.00	2.00
Baer and Kates (20)					
	di-16:0	0.96	2.00	1.00	2.00

The TLC behaviour of the synthetic PSC's was identical to that of the hydrogenated PSC from N. alba (Fig. 14). The mobilities on TLC (Rf) were: 0.38 ± 0.01, 0.29 ± 0.02 and 0.38 ± 0.01 in solvent systems: chloroform:methanol:ammonia (65:35:5, by vol.), chloroform:methanol:water (65:36:5, by vol.) and chloroform:methanol:acetic acid:water (80:20:4:2, by vol.) respectively. Both the natural and the synthetic derivatives of PSC stained positively with the phosphate stain (blue) and with the Dragendorff reagent (orange).

An almost straight line function was obtained from a graph of the specific optical rotations of the saturated PSC's versus their fatty acid chain length (Fig. 15). A similar trend was seen with the corresponding synthetic PC's (20). The saturated synthetic PSC's had fairly constant molecular rotations, in the range of +56-58 °, identical in sign and magnitude to that of hydrogenated PSC from N. alba (M_D, +53 °). The high optical rotations (Fig. 15) indicated that the synthetic PSC's had a high degree of optical purity. The natural and synthetic saturated PC's had somewhat lower molecular rotations (M_D, +49-50°) (20).

These results establish unambiguously the sn-3 configuration of the PSC from N. alba. The same configuration is generally found for all glycerophospholipids with the exception of the phytanyl ether-linked phospholipids of the archaebacteria (extreme halophiles, methanogens and thermoacidophiles) (78) and the lyso bis-phosphatidic acid found in the lysosome of mammalian cells (79) which have the opposite sn-1 configuration.

1.6.2.1 Thermal behaviour of PSCs

In general, anhydrous PSC's show thermal behaviour similar to that found for PC. When DPPSC was heated in a melting point tube the solid white amorphous powder of the saturated DPPSC underwent a transition at 80 °C. The substance became translucent but did not liquify as there was no formation of a meniscus. This transition is not a true melting but is probably characteristic of solid-liquid-crystalline transitions. On further heating to about 100°C the compound decomposed spontaneously. By contrast the corresponding PC, DPPC forms a meniscus at 225-226 °C and decomposes at much higher temperature (20).

Transition temperatures were also measured by DSC (Fig. 16). The saturated synthetic PSC's showed fairly sharp transition temperatures (over a range of 2-3 °C) with onset of melting at 55 °C for di-14:0 PSC, 81 °C for di-16:0 PSC and 87 °C for di-18:0 PSC (Figs 16 A, 16 B and 16 C respectively). By contrast, the corresponding PC's showed onset of melting at lower temperatures: 42 °C for di-14:0 PC, 69 °C for di-16:0 PC and 81 °C for di-18:0 PC (Figs 16 A, 16 B and 16 C respectively). Thus each of the PC's showed a lower transition temperature than the corresponding PSC, the difference being inversely proportional to the fatty acid chain length. For both PC and PSC the transition temperatures increased with increasing fatty acid chain length in similar fashion, the increase between the di-16:0 and the di-14:0 homologues being greater than between the di-16:0 and the di-18:0 homologue.

Figure 14: TLC of A) egg-PC, B) egg-PSC and C) PSC from N. alba

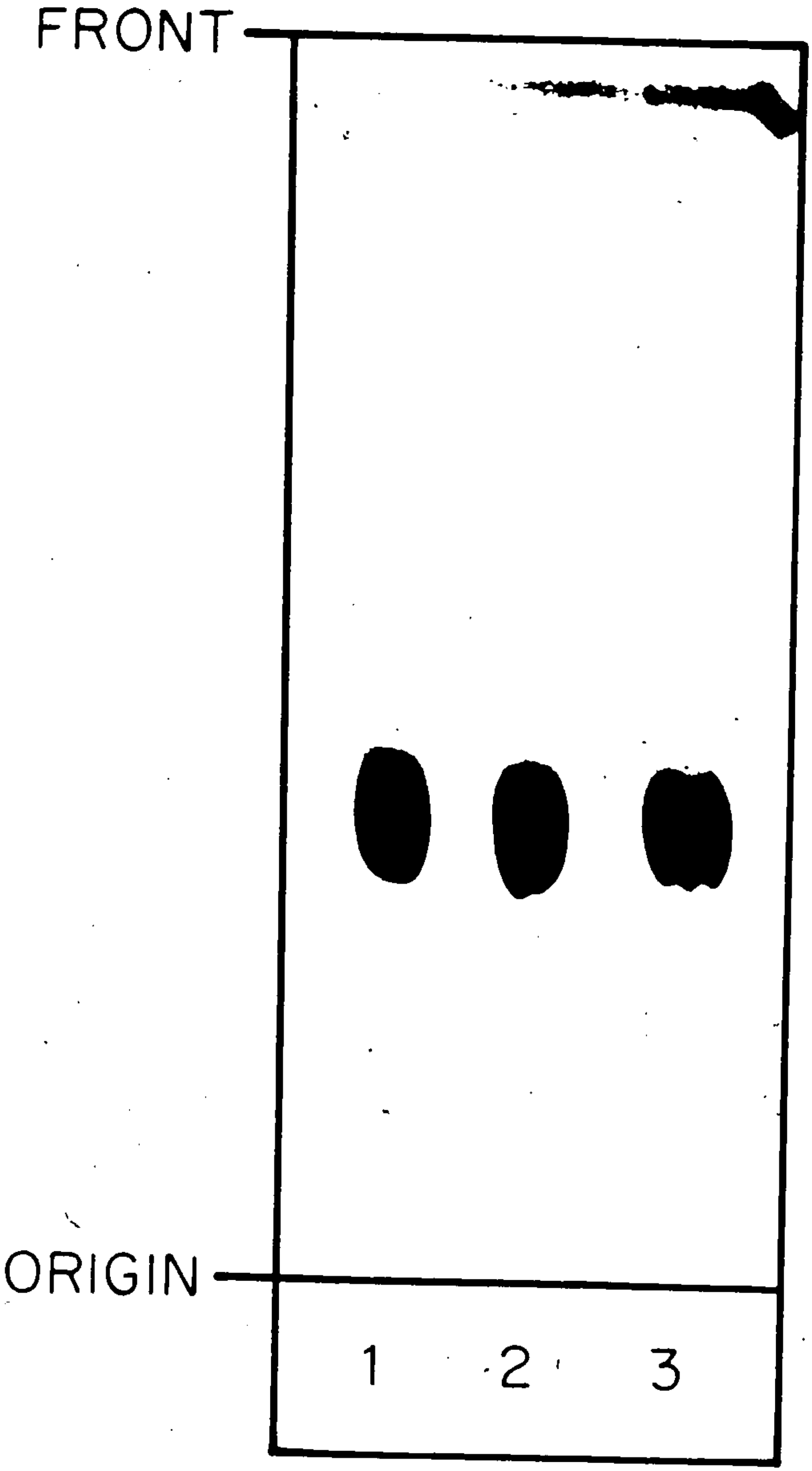
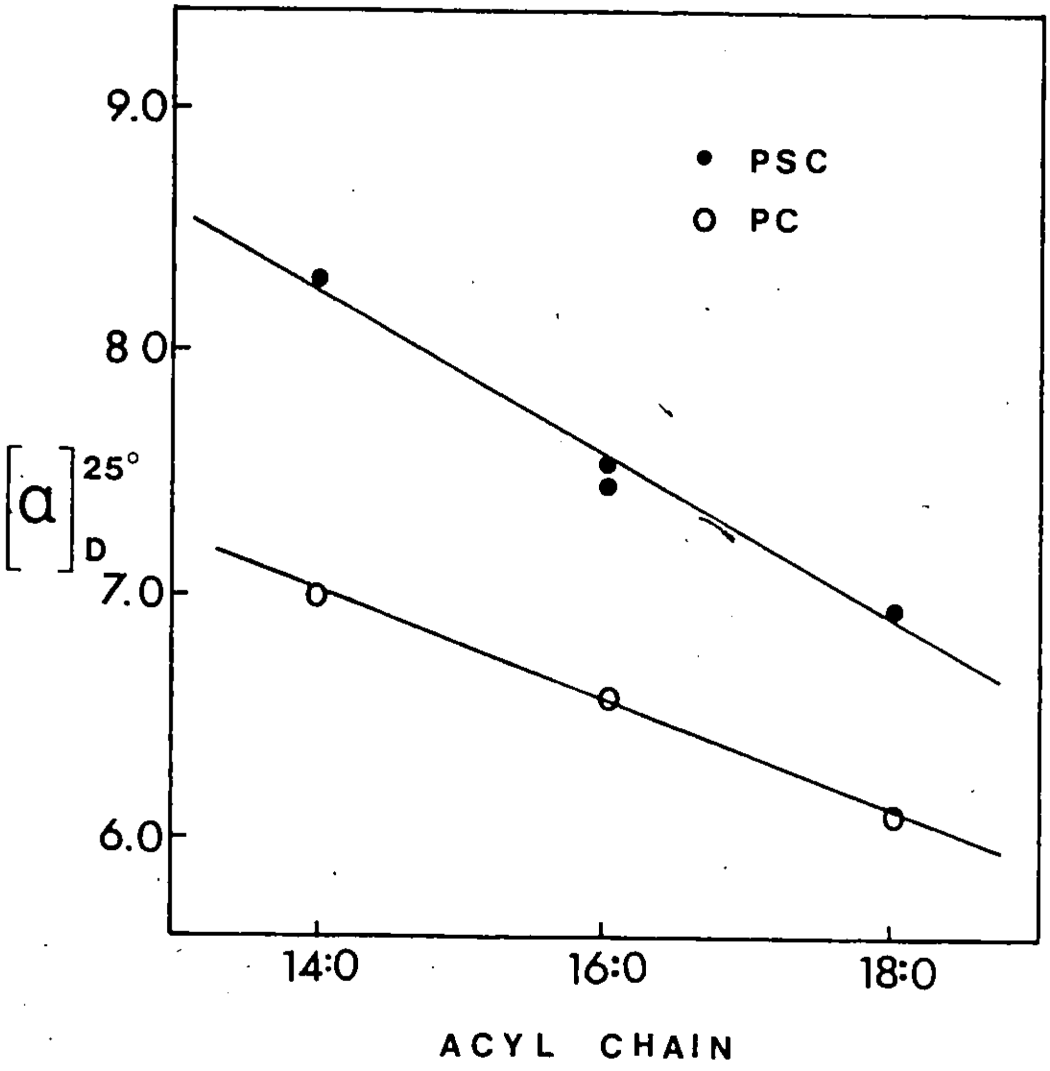
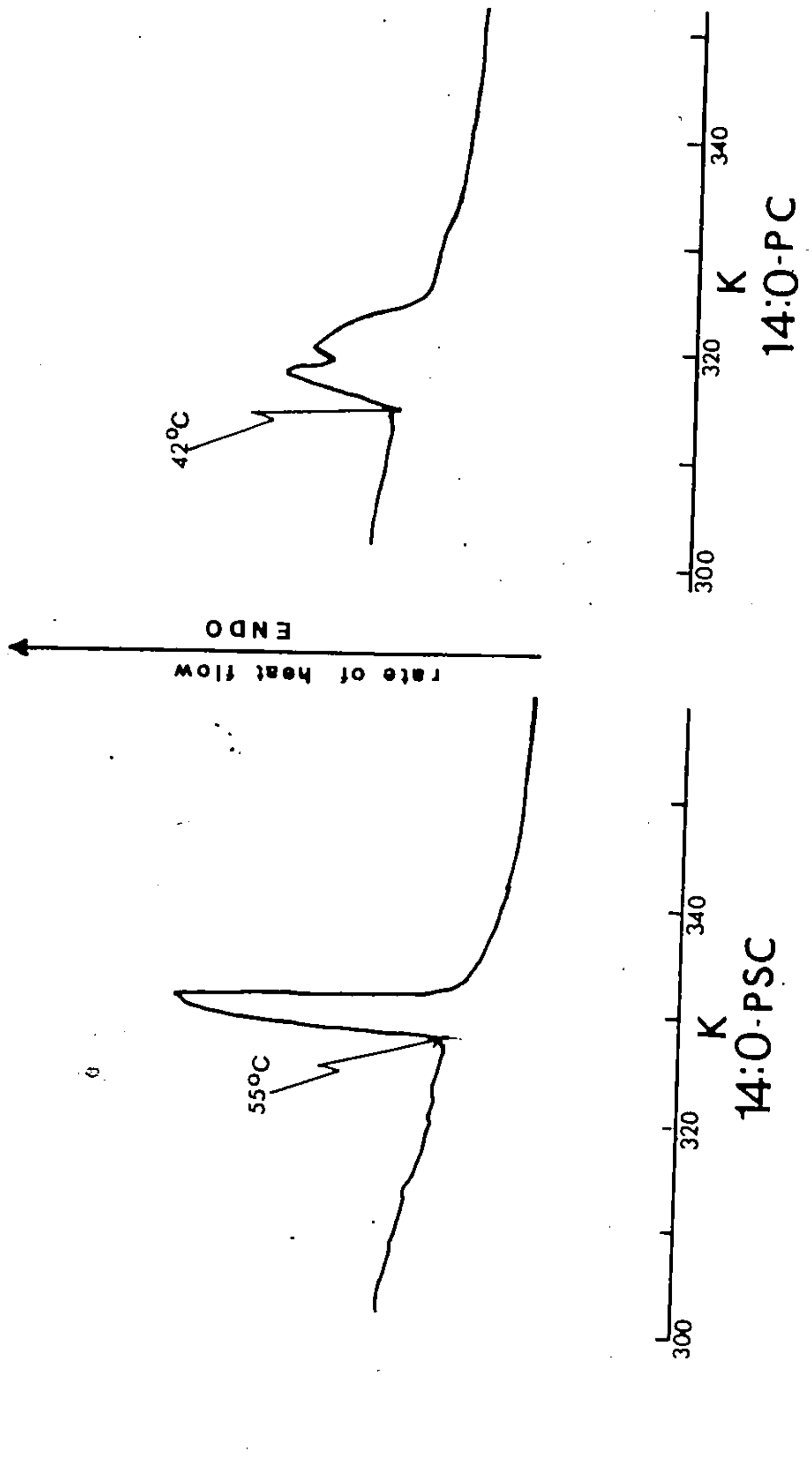

FRONT
ORIGIN
1
2
3

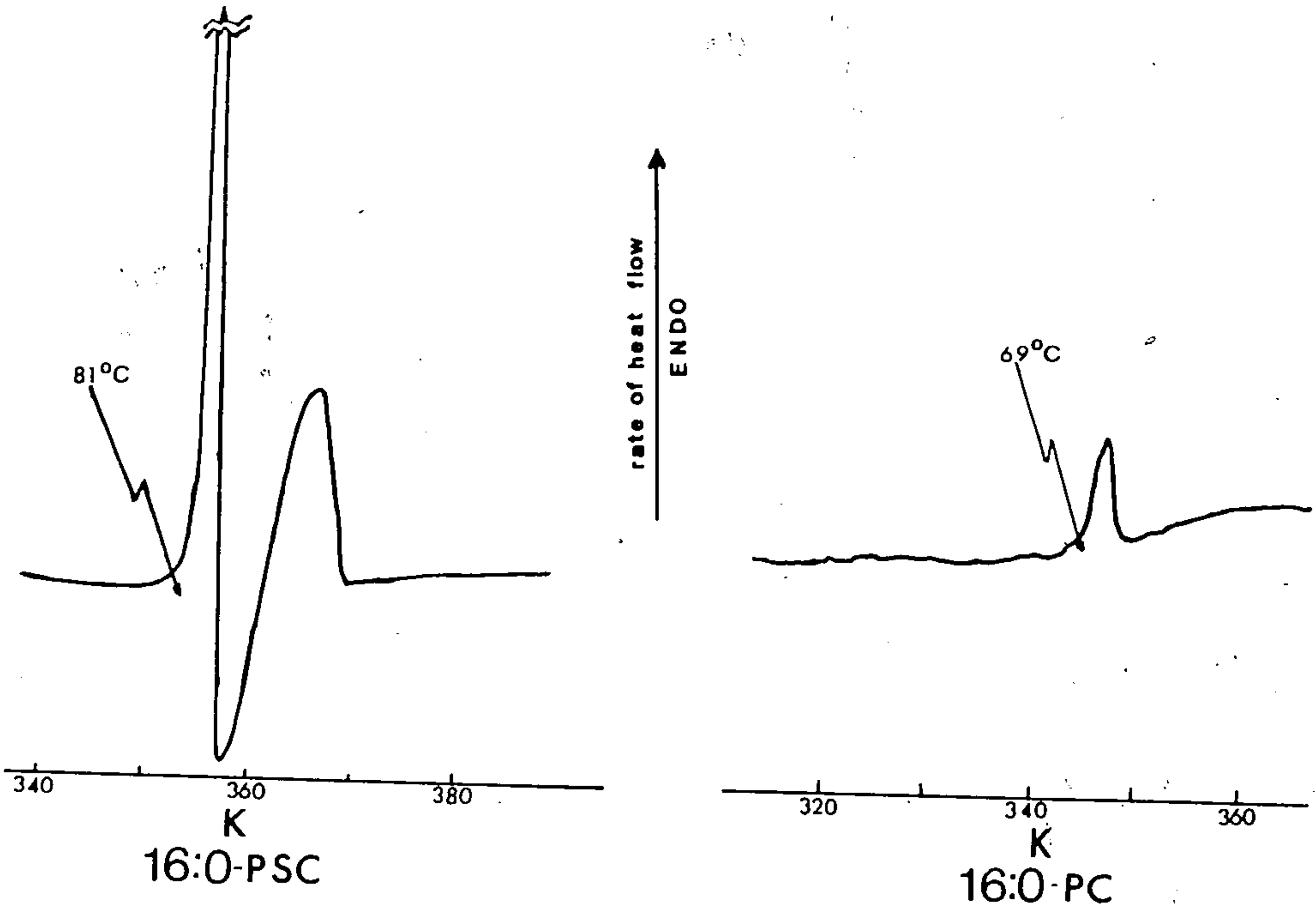
Figure 15: FA chain length and the specific rotation. The $[\alpha]_D$ of the PSCs were measured in chloroform:methanol (1:1, v/v). PCs data is from 20

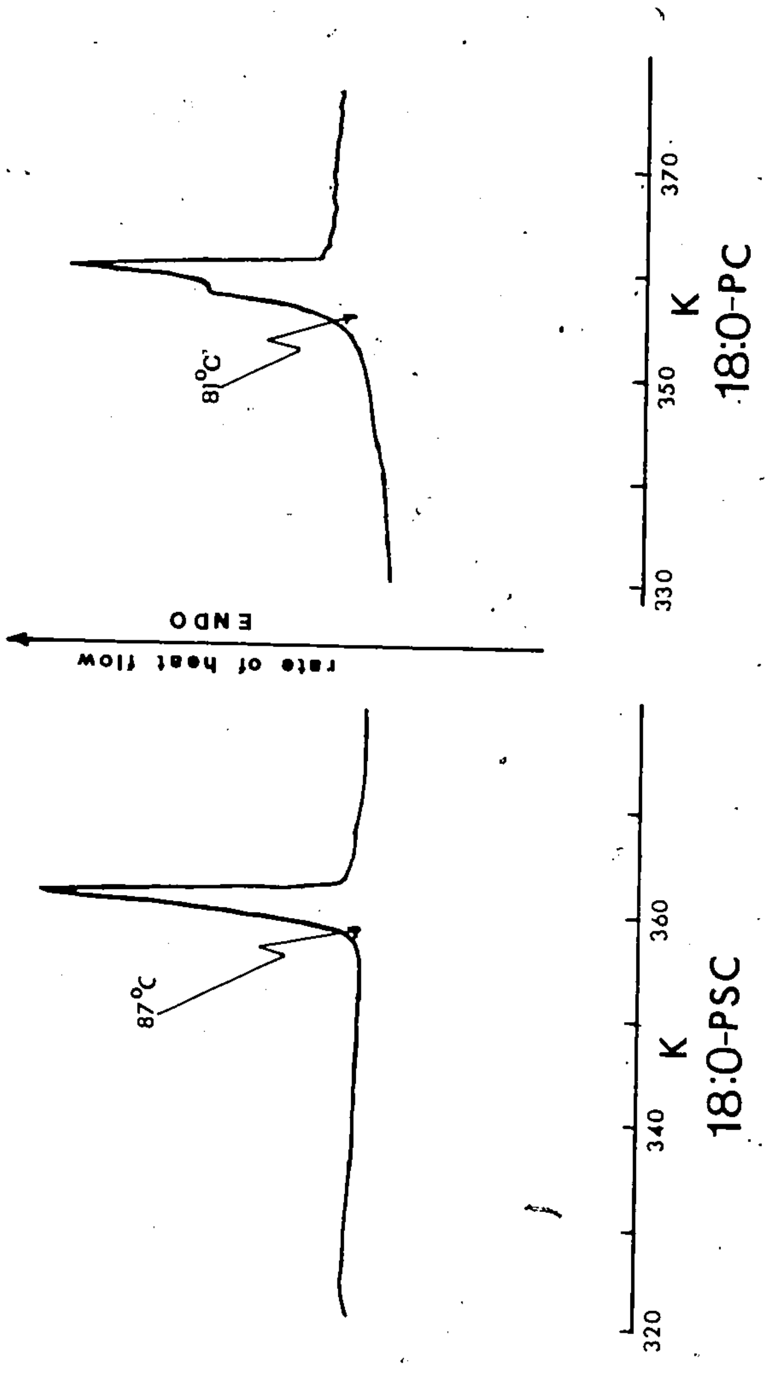


9.0
8.0
7.0
6.0
$[\alpha]_D^{25°}$
● PSC
O PC
14:0
16:0
18:0
ACYL CHAIN

Figure 16: DSC curves of anhydrous saturated PSCs and PCs


ENDO
rate of heat flow
42°C
300
320
340
K
14:0-PC
55°C
300
320
340
K
14:0-PSC


rate of heat flow
ENDO
81°C
340
360
380
K
16:0-PSC
69°C
320
340
360
K
16:0-PC

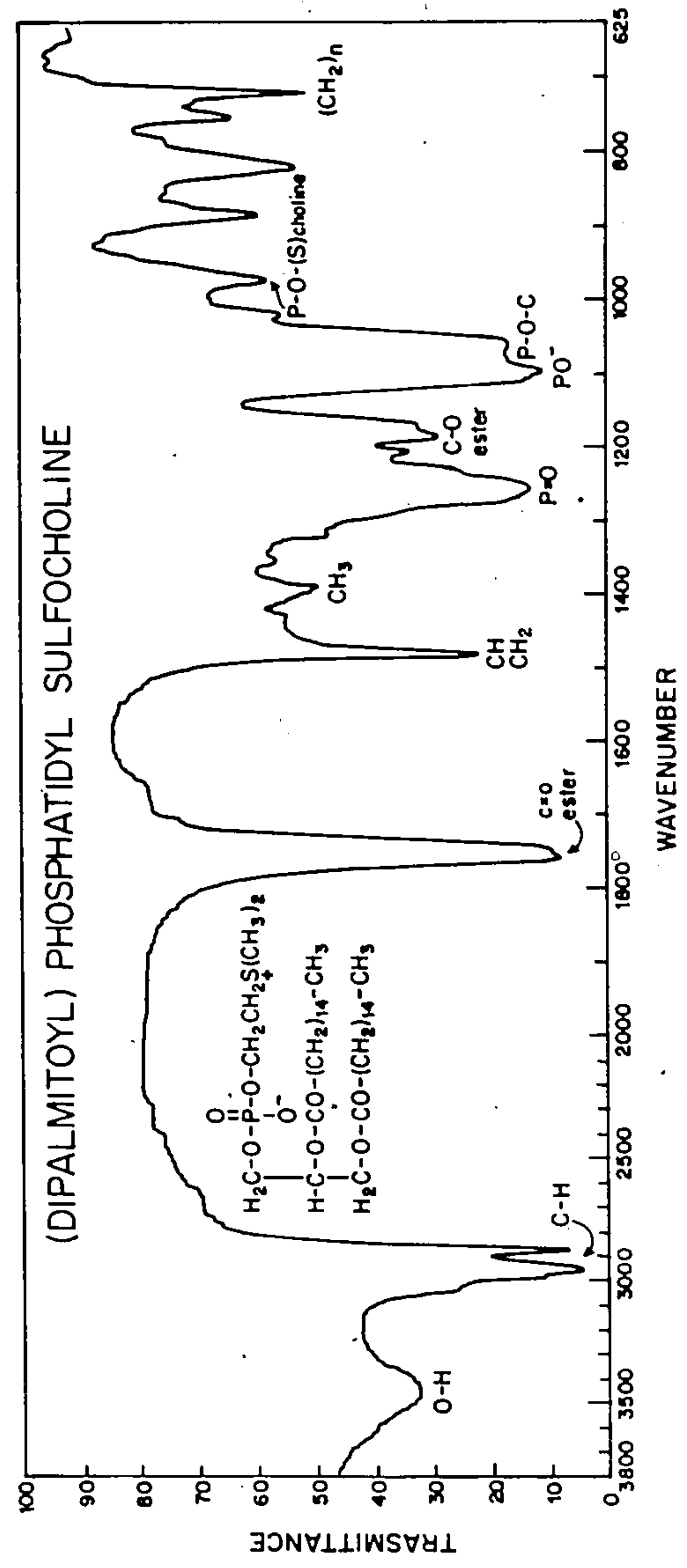

18:0-PC
K
330
350
370
81°C
rate of heat flow
ENDO
18:0-PSC
K
320
340
360
87°C

1.6.3 Spectroscopic analysis of PSC's

1.6.3.1 Infrared Spectroscopy

The infrared spectra of the saturated PSC (Fig. 17) were identical in all respects to the hydrogenated PSC from *N. alba*. A striking similarity with the spectra of saturated PC's was also noted, except for the absence of the medium strength band at 970 cm^{-1} which is characteristic of the quaternary ammonium $(CH_3)_3N^+$ group of PC's. The spectra of the PSC's (KBr pellet) showed the expected absorption bands at 3450 cm^{-1} (H-bonded OH), 2990, 2958, 2875 cm^{-1} (CH_3, CH_2, asymmetric stretching), 1750 cm^{-1} (ester C=O, stretch), 1482 cm^{-1} (CH_2, bend), 1430 cm^{-1} (CH_3, asymmetric deformation), 1390 cm^{-1} (CH_3, symmetric deformation), 1265 cm^{-1} (P=O, stretch), 1190 cm^{-1} (ester C-O, asymmetric stretch), 1095 cm^{-1} {$O-P(O)_2$ -O} antisymmetric PO^- stretch) and several weak absorptions at 1020 cm^{-1} (CO-P, stretch), 975 cm^{-1} ($C-S^+-C$, stretch), 885 cm^{-1} (C-C, stretch), 820 cm^{-1}, 755 cm^{-1} and 722 cm^{-1} {$(CH_2)n$, rock}. Because of the similarity between the infrared spectra of PC's and the PSC's, this technique is not suitable for distinguishing the two. The peak at 970 cm^{-1} is the only distinguishing absorption. However the Fourier transform infrared spectra of aqueous dispersions of DMPSC showed additional diagnotic absorption bands at 3024 cm^{-1} (CH_3, asymmetric stretch) and 1435 cm^{-1} {$S^+(CH_3)_2$, asymmetric CH_3 deformation} (80).

Figure 17: Infrared spectrum of synthetic 1,2-dipalmitoyl-<u>sn</u>-3-phosphatidyl sulfocholine in KBr


(DIPALMITOYL) PHOSPHATIDYL SULFOCHOLINE
TRASMITTANCE
WAVENUMBER
0
10
20
30
40
50
60
70
80
90
100
3800
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625
O-H
C-H
C=O ester
CH
CH2
CH3
P=O
C-O ester
P-O-C
PO⁻
P-O-(S)choline
(CH2)n
H2C-O-P(=O)(O⁻)-O-CH2CH2S(CH3)2+
H-C-O-CO-(CH2)14-CH3
H2C-O-CO-(CH2)14-CH3

1.6.3.2 Nuclear Magnetic Resonance

A Proton NMR

The proton NMR spectra of dipalmitoyl phosphatidyl sulfocholine (Fig. 18) and the natural hydrogenated PSC from N. alba were identical (Table 2). The spectra of the natural unhydrogenated PSC differed only in that it showed the presence of unsaturated methyne signals of the fatty acid side chains. The spectra of DPPC and DPPSC were also very similar except for small differences located in the signals for the head groups. Thus the diagnostic groups in PMR spectra are: $S(C\underline{H}_3)_2$ at 3.2 (singlet, 6H), $SC\underline{H}_2$ at 3.95 (multiplet, 2H) and $P\text{-}O\text{-}C\underline{H}_2$ (S-choline) at 4.36 (multiplet, 2H). Those of the corresponding PC are: $N(C\underline{H}_3)_3$ at 3.4 δ, $NC\underline{H}_2$ at 3.8 and $POC\underline{H}$ (N-choline) at 4.36. These differences can be used for the identification of sulfonium analogues of PC isolated from natural sources. This is shown in the NMR spectra of 1:1 and 1:2 mixtures of PSC and PC (respectively) where the methyl singlets at 3.4 and 3.2 can be clearly distinguished from one another (Fig. 19).

Figure 18: 100 MHz proton NMR spectrum of synthetic 1,2-dipalmitoyl-*sn*-3-phosphatidyl sulfocholine in $CDCl_3$.

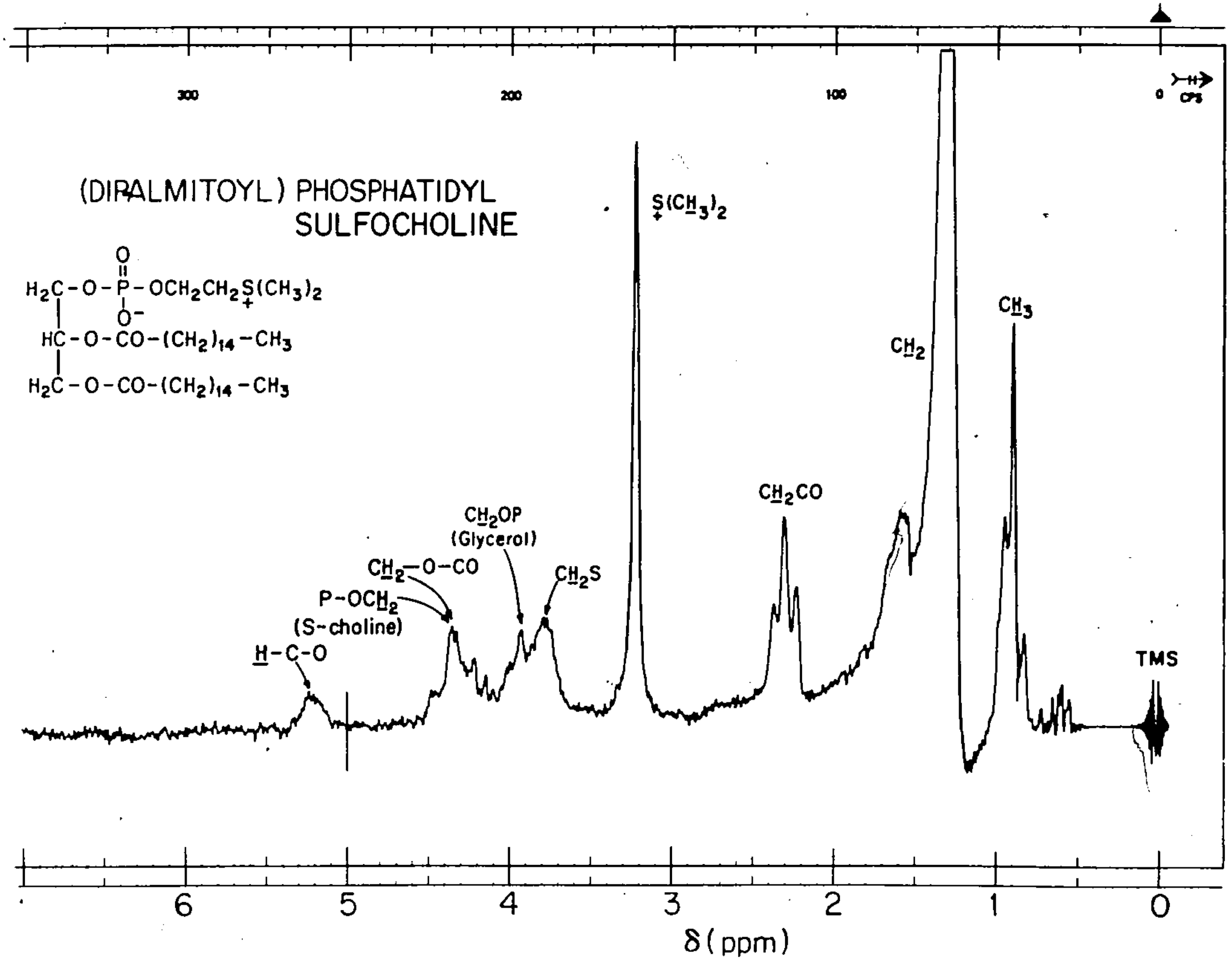
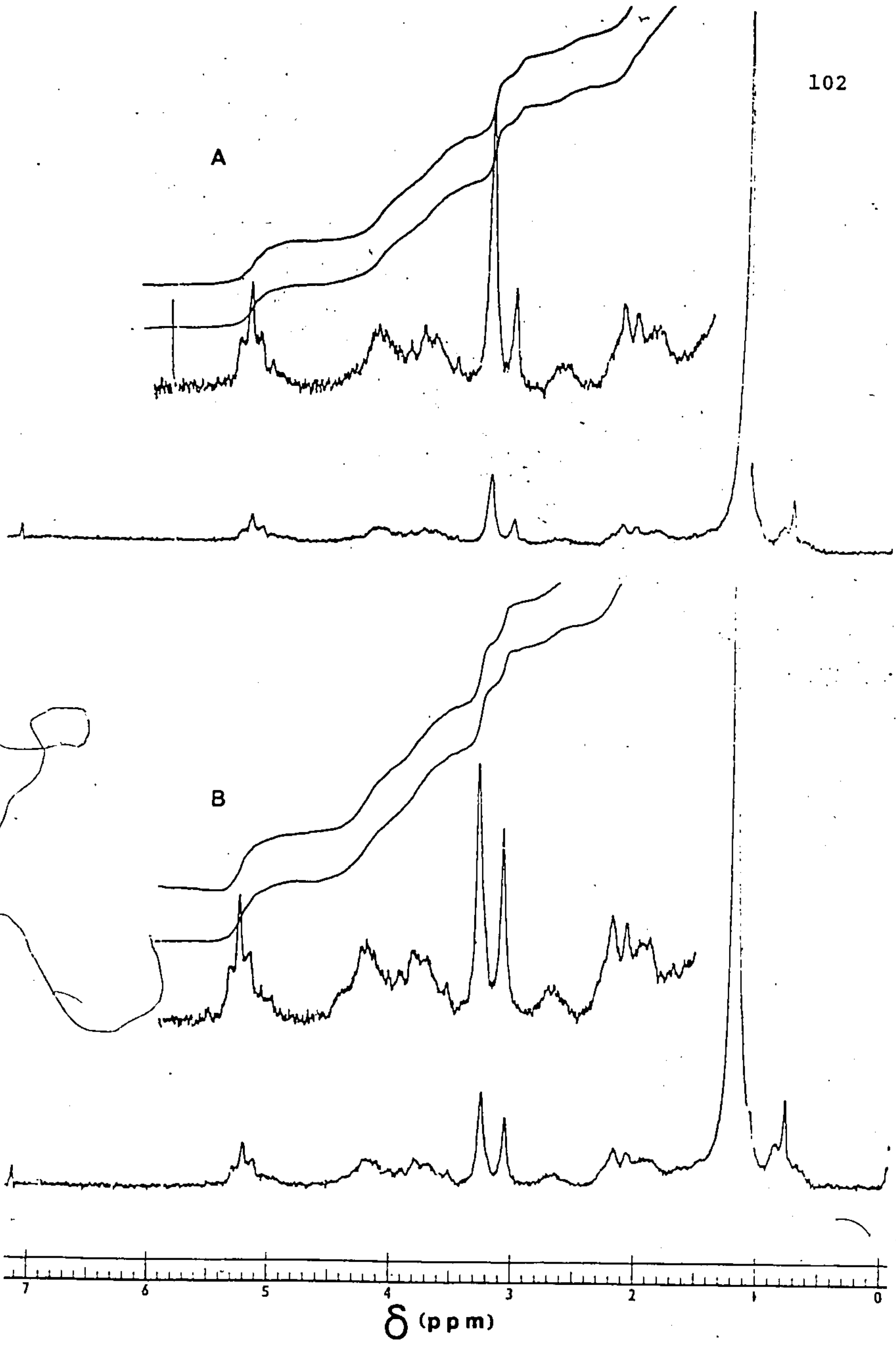

(DIPALMITOYL) PHOSPHATIDYL
SULFOCHOLINE
H2C-O-P(=O)(O−)-OCH2CH2S+(CH3)2
HC-O-CO-(CH2)14-CH3
H2C-O-CO-(CH2)14-CH3
0 CPS
100
200
300
S+(CH3)2
CH3
CH2
CH2CO
CH2OP (Glycerol)
CH2S
CH2-O-CO
P-OCH2 (S-choline)
H-C-O
TMS
6
5
4
3
2
1
0
δ (ppm)

Figure 19: 100 MHz PMR spectra of 1:1 (bottom) and 1:2 (top)(mole:mole) mixtures of egg-PSC and egg-PC, respectively.


A
B
7
6
5
4
3
2
1
0
δ (ppm)

B ^{13}C NMR

The ^{13}C NMR spectra of synthetic DPPSC (Fig. 20) and of the natural hydrogenated PSC from N. alba (Fig. 21) were identical (Table 3). Of particular interest are the diagnostic signals in the spectra assigned to the sulfonium group: $S-(C\underline{H}_3)_2$ at 45.7 δ, $S-C\underline{H}_2$ at 63.7δ and $P-O-C\underline{H}_2$ (S-choline) at 59.4 and 59.9 δ.

These signals as well as the glycerol carbon signals were quite broad (Fig. 20) relative to those of the hydrocarbon groups. The probable explanation for this is the formation of inverted micelles in non-aqueous solvents such as chloroform. The polar head groups are internalized and immobilized through hydrostatic bonding between the positive sulfonium and the neighbouring phosphate groups. The fatty acid side chains on the other hand project freely into the chloroform solvent with little or no inter-chain van der Waals attractions between them. The T1 relaxation times of rigid and immobile groups is long compared to the relatively short T2 and produces broad signals while highly mobile groups have much longer T2 relative to T1 and therefore give sharp signals. In addition, the broadening of the peaks is partially due to the long range phosphorus splitting. This can be clearly seen in the doublet at 72 δ.

The ^{13}C spectra are less important for diagnostic purposes. ^{13}C NMR requires large samples of over 100 mg and long measuring times (1 hour or more).

Figure 20: ^{13}C-NMR spectrum of synthetic 1,2-dipalmitoyl-sn-3-phosphatidyl sulfocholine in $CDCl_3$.

(DIPALMITOYL) PHOSPHATIDYL SULFOCHOLINE

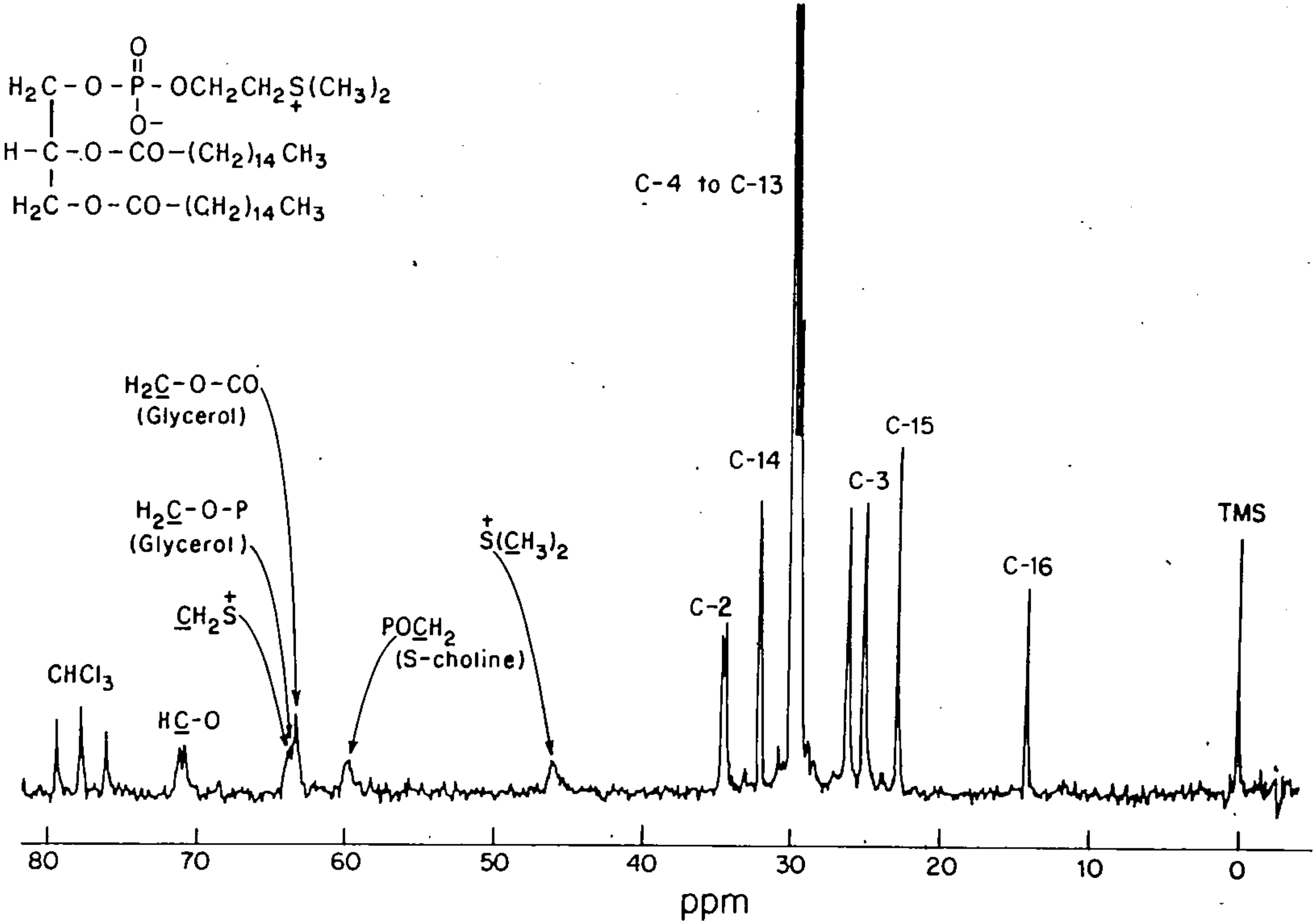
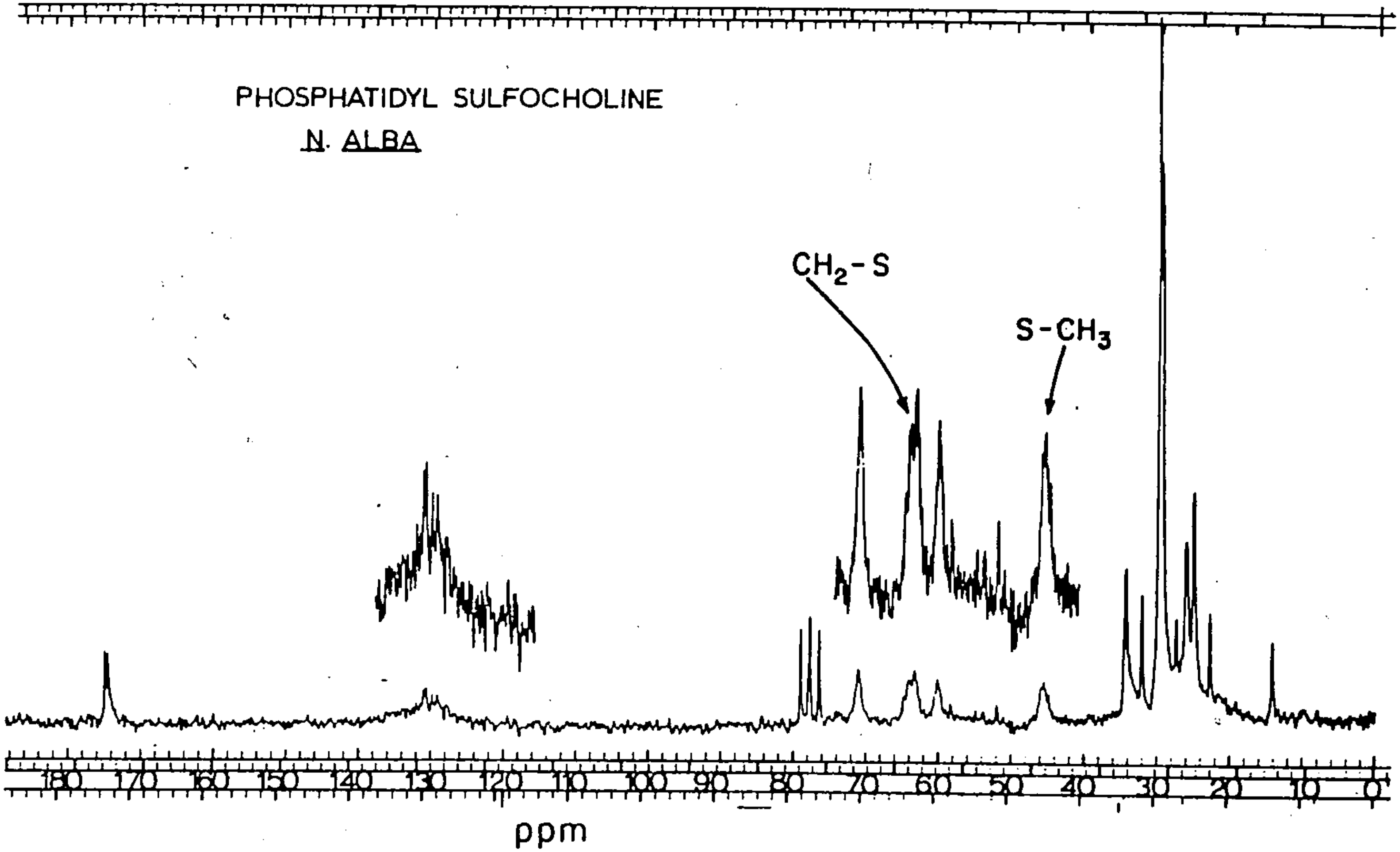


Figure 21: ^{13}C-NMR spectrum of synthetic 1,2-dipalmitoyl-sn-3-phosphatidyl sulfocholine from *N. alba* in $CDCl_3$.



PHOSPHATIDYL SULFOCHOLINE

N. ALBA

TABLE 2

^{1}H-nmr Assignments For Phosphatidyl Sulfocholine

GROUP ASSIGNMENT	SYNTHETIC*			NATURAL**	
	δ (ppm)	No. of Protons Found	Calc.	δ(ppm)	No. of Protons
HCO glycerol (multiplet)	5.20	1.0	1.0	5.20	1.0
CH_2-CO-R	4.36	4.0	2.0	4.36	2.0
CH_2-O-P(O)[S-choline]			2.0		2.0
CH_2-O-P(O) [glycerol]	3.90	4.5	2.0	3.90	2.0
CH_2-S^+ [multiplet)]			2.0	3.80	2.0
$(CH_3)_2S^+$ [singlet]	3.21	6.0	6.0	3.20	6.00
CH_2CO [triplet]	2.28	3.9	4.0	2.28	4.0
CH_2 [multiplet]	1.28	47.5	52.0	1.28	58.0
CH_3 [triplet]	0.90	6.0	6.0	0.90	6.0

* (Dipalmitoyl) phosphatidyl sulfocholine

** (Hydrogenated *N. alba* phosphatidyl sulfocholine (major acyl groups, 14:0, 18:0 and 20:0)

TABLE 3

^{13}C-nmr Assignment For Phosphatidyl Sulfocholine

GROUP ASSIGNMENT	SYNTHETIC*		NATURAL**
	δ (ppm) (Rel to TMS)	No of C-atoms	δ (ppm) (Rel to TMS)
Acyl C-16, Terminal methyl	14.12	2	14.08
Acyl C-15, Adjacent to terminal methyl	22.75	2	22.68
Acyl C-3	25.01	2	25.04
	26.11		26.00
Acyl C-4 to C-13	29.28 29.44 29.78	18	29.7
Acyl C-14	32.00	2	32.00
Acyl C-2 (_sn_-1 position)	34.22	1	34.32
Acyl C-2 (_sn_-2 position)	34.41	1	
$(CH_3)_2$ S^+	45.70	2	45.56
$POCH_2$ (S-choline)	59.42	1	59.93
H_2C-O-CO- (_sn_-1-glycerol)	62.93	1	62.70
H_2COP (_sn_-3-glycerol)	63.41	1	63.45
CH_2S^+ (sulfocholine)	63.65	1	63.72
HCO (_sn_-2-glycerol)	70.60	1	70.40
C=O (_sn_-1-position)	173.08	1	173.20
C=O (_sn_-2-position)	173.47	1	173.60

* (Dipalmitoyl) phosphatidyl sulfocholine

** Hydrogenated _N. alba_ phosphatidyl sulfocholine (14:0,18:0 and 20:0 acyl groups)

1.6.4 Mass Spectrometry of PSC

The field desorption mass spectra of the PSC homologues (Table 4) gave base ion peaks corresponding to the theoretical molecular weigh, M + a proton $(M+H)^{+}$ with m/z (mass to charge ratio) of 681, 737, 793, 789 for di-14:0, di-16:0, di-18:0, and di-18:1 PSC's, respectively.

The spectra of all samples showed the expected ions from the isotopes of C and S ($M+2^{+}$ and $M+3^{+}$) with intensities slightly higher than calculated (81, 82). Several peaks were noted that had higher molecular weights than the base peak, which is thought to be due to the formation of complexes between a molecule of PSC and one of the intense polar head fragments (e.g. m/z: 89, 107, 187). Thus, M+15 or $M+(CH_3)$ ions were prominent in the spectra of all samples and are thought to arise from the intermolecular transfer of sulfonium methyl groups, analogous to the methyl transfer demonstrated for PC (82). Di-14:0 PSC showed other heavy ion complexes at m/z 755 (M+75) while the heavier ions of di-16:0 were measured at m/z 797, 811, and 826 (M+61, M+75, M+90, respectively). The following structures may be the fragments transferred to form the heavier complexed ions: CH_3-S-CH_2- (M+61), CH_3-S-CH_2-CH_2- (M+75), $(CH_3)_2S^{+}$-CH_2-CH_2- (M+90). These ions were weak or absent in the spectra of the heavier derivatives, di-18:0 PSC and di-18:1 PSC.

Several ions of low molecular weight were seen in the spectra of all PSCs, corresponding well with the polar head group phosphosulfocholine (m/z 187), sulfocholine (m/z 107), dehydrated sulfocholine (m/z 89) and the diacyl glycerol moiety (R$^{\cdot}$, Table 4). Other diagnostic peaks can be assigned to the following fragments: M-61 (M+H- $(CH_3)_2$-S-), M-59 (M+H- CH_3-S-CH_2+ H), and M-45 (M+CH_3-CH_2-S-CH_3+ H). The assignment of these fragments is questionable because of the several complexing steps required (82). In a few spectra (e.g. di-16:0 PSC and the natural mixture of PSCs from N. alba) the following ions were observed: m/z 77 and 121. These ions correspond to $(CH_3)_2$-S$^{\cdot}$ and methoxy sulfocholine, respectively.

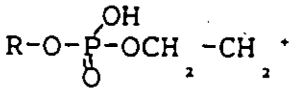
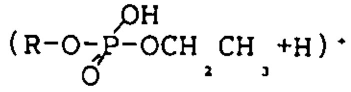
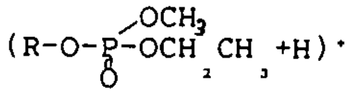
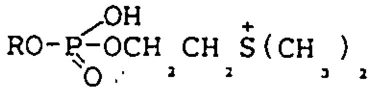
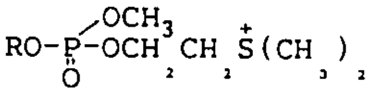
The spectra reported here are very similar to the spectra of the hydrogenated natural PSC from N. alba, and the assignments of the present data are also consistent with those reported for the hydrogenated molecular species of PSC from N. alba (6).

Deuterated derivatives

Two deuterated derivatives, one with a deuterated head methyl and the other with the C2 position of the palmitic acid deuterated were synthesized. The mass spectra (not shown) showed the expected ion peaks together with other ion peaks which could not be assigned unambiguously because of scrambling of the deuterium atoms.

TABLE 4

Principal Ions in Field Desorption Mass Spectra of PSCs

Fragment	Tentative Assignment	14:0 PSC		16:0 PSC		18:0 PSC		18:1 PSC	
		m/z	RI%	m/z	RI%	m/z	RI%	m/z	RI%
89	$CH_2{=}CH{-}\overset{+}{S}(CH_3^{\cdot})_2$	89	21	89	14	89	1	89	51
107	$HOCH_2{-}CH_2{-}S^{+}(CH_3)_2$	107	4	107	20	107	-	107	2
187	$(HO)_2POCH_2CH_2\overset{+}{S}(CH_3)_2$	187	1	187	1	187	1	187	-
M-185	R+ (Footnote b)	495	5	551	11	607	3	603	-
M-184		496	3	552	11	608	-	604	-
M-61	 $R{-}O{-}\overset{OH}{\underset{O}{P}}{-}OCH_2{-}CH_2^{+}$	619	4	675	5	731	4	727	-
M-60		620	-	676	-	732	2	728	4
M-59	 $(R{-}O{-}\overset{OH}{\underset{O}{P}}{-}OCH_2CH_3+H)^{+}$	621	1	677	11	733	-	729	6
M-58		622	-	678	6	734	-	730	2
M-46	 $(R{-}O{-}\overset{OCH_3}{\underset{O}{P}}{-}OCH_2CH_3+H)^{+}$	634	-	690	-	746	5	742	-
M-45		635	1	691	10	747	3	743	1
M-1	 $RO{-}\overset{OH}{\underset{O}{P}}{-}OCH_2CH_2\overset{+}{S}(CH_3)_2$	679	5	735	10	791	6	787	-
M		680	2	736	-	792	-	788	5
M+1		681	100	737	100	793	100	789	100
M+2		682	52	738	53	794	54	790	17
M+3		683	7	739	15	795	-	791	5
M+14	 $RO{-}\overset{OCH_3}{\underset{O}{P}}{-}OCH_2CH_2\overset{+}{S}(CH_3)_2$	694	-	750	-	806	-	802	3
M+15		695	13	751	32	807	19	803	48
(M+1)+15		696	6	752	12	808	21	804	26
M		680.4		736.5		792.6		788.5	
anode current (mA)		10-13		18		16		17-19	
number of scans		4		1		1		5	

1.7 DISCUSSION

The above results confirm the successful synthesis of several optically active homologues of phosphatidyl sulfocholine the major phospholipid of *N. alba*. The pure synthetic PSC's were obtained in high yields (50-70% by the Aneja method (38)), and both saturated and unsaturated monoacid, mixed acid and deuterated derivatives of PSC were prepared. The synthetic procedure could also be used for the synthesis of radioactive PSC.

It is interesting to note that PSC may occur in other organisms other than *Nitzschia alba*. Maw and de Vignaud (47) have shown that sulfocholine fed to rats is incorporated into the phospholipids of the liver. Later the work of Bjerve and Bremer (14) using ^{35}S and ^{3}H labelled sulfocholine confirmed this and showed that the radioactive (^{35}S and ^{3}H) phospholipid co-chromatographed with PC on TLC. Kates and Volcani (4) also demonstrated the presence of a sulfur containing lipid that co-chromatographed with lecithin from 5 different species of marine diatoms. Anderson and Bilan (13) have achieved an almost complete replacement of PC by PSC in cultured LM cells. The cells were grown with ^{35}S-labelled sulfocholine as the sole choline source. Similar results have been obtained with yeast cells and with the diatom *N. alba* itself (63).

These findings together show that PSC can indeed replace PC in biomembranes of many types of living cells. The question now arises concerning the structure-function relationships of the sulfonium analogues as membrane components compared to the ubiquitous phosphatidyl choline. It is this question which is investigated in Part II of this thesis.

PART II

PHYSICAL STUDIES OF PSC

2.1 INTRODUCTION

The presence of phosphatidyl sulfocholine (PSC) as a major membrane component in diatoms (1) and in LM cells (13) and in yeast cells cultured in the presence of sulfocholine (63) raised questions regarding the effect of this sulfonium lipid on the physical and dynamic properties of the respective biomembranes. Some of these questions are:

1. What are the polymorphic forms of PSC?
2. What is the thermal behaviour of PSC?
3. What is the effect of the sulfonium head group on the fluidity of PC?
4. Does PSC interact with cholesterol as does PC?
5. What is the effect of PSC on the permeability of membranes?

Having at our disposal a homologous series of synthetic phosphatidyl sulfocholines, we undertook a comparative study of the physical properties of aqueous dispersions of the sulfonium and ammonium analogues of lecithin with the view of answering these questions. Lecithin-water dispersions have been commonly used as model membrane systems in studies of structure-function relationships of biological membranes. The effect of the fatty acid side chain on membrane fluidity is well recognized and has been widely studied (83), but attention is shifting presently towards the effect of the polar head group on the properties of biomembranes (87, 83, 85).

The question of thermal behaviour and the effect of polar head on fluidity was investigated by several techniques, such as, differential scanning calorimetry (DSC), steady state fluorescence polarization, electron paramagnetic resonance (EPR), deuterium and phosphorus broad band nuclear magnetic resonance and fourier transform infrared spectroscopy (FTIR). The morphology of PSC dispersions was examined by freeze-etch electron microscopy , phase contrast light microscopy, ^{31}P-NMR and monolayer studies. The interaction of PSC with cholesterol was studied by DSC and permeability (towards urea) in presence and absence of cholesterol was measured by osmometry (not presented in this thesis).

Before proceeding with the presentation of the results of these studies a brief review of the knowledge of our physical properties of natural and model membrane systems, followed by a review of the physical methods used in the present study will be given.

2.2 PHYSICAL PROPERTIES OF NATURAL AND MODEL MEMBRANES

Phospholipids exhibit complex morphological behaviour which is affected by the structure of the phospholipid, the solvent environment, the solutes present and finally the method of preparation of the lipid dispersions. Their morphology and fluidity is also greatly influenced by temperature (thermotropic effect) (86, 87). These points were studied with respect to the effect of the sulfonium polar head and are therefore discussed below, in the following sections.

2.2.1 Mesomorphic Phases (89)

The behaviour of phospholipids in water is dependent on its amphipathic nature. The polar head moiety of these amphipaths is soluble in water because of the strong polar bonding between the charged head group and water. The fatty acid tail however excludes water for the following reasons based on the thermodynamic consideration of the 'hydrophobic effect' (88): water is a highly structured material with an extensive hydrogen bonding network between the water molecules; the interaction of polymethylene groups with water molecules necessitates a restructuring or distortion of the structure of water. No favourable bonding can occur between water and hydrocarbon chains to compensate for the loss of H-bonds in water. In fact, the introduction of acyl chains into an aqueous medium requires an unfavorable loss in entropy for the system. These two factors render the process energetically very unfavorable and leads to the exclusion of hydrocarbon from water. The free energy for exclusion of a hexadecane from water into a non-polar medium has been calculated to be between -850 to -600 cal/mole (88). This gives an equilibrium constant of 10^7 in favor of the exclusion of water. Thus phospholipids in aqueous media spontaneously form aggregate structures (i.e. <u>mesomorphs</u>) (Fig. 22), as has been amply documented (89).

The dry or monohydrated saturated lecithins exist in several crystalline states but lecithins precipitated from acetone show a reproducible 'simple' transition at a temperature somewhat higher than the transition temperature of the fully hydrated lecithins. As one adds water to the monohydrated lecithin sample, there is a progressive lowering of the transition temperature to a stable temperature called the gel to liquid-crystalline transition temperature (Tm) (2). Further dilution with water to a solute concentration of 10^{-10} M (the critical micelle concentration, CMC) produces no change in the transition temperature nor in the enthalpy of transition, the lecithin remaining in an aggregated form. Below this concentration the lecithin ceases to exist in an organized or aggregated form (liposome or vesicle) and instead behaves as freely soluble molecules (2).

A variety of lipid aggregate structures are known such as: the monolayer, the micelle, the bilayer and the hexagonal phases (Fig. 22) (see reviews: 90, 89, 91).

2.2.1.1 The Monolayer

Amphipathic molecules spread as a monolayer film on the surface of water with the hydrophilic head group submerged in the water and the fatty chains extended upward in the air. This system resembles a two dimensional gas, the molecules cover the entire area and act more or less as independent molecules. If the area is diminished, the molecules on the surface will be forced to come together. The presence of a lipid layer on the surface of water lowers the surface tension of the water. The more condensed the lipids, the lower the surface tension. From the measurement of this property, the events occurring in the monolayer can be monitored. Upon compressing the film the mobility of the surface head groups is restricted first, and finally, the fatty acyl tails are immobilized perpendicular to the surface of the monolayer. At the highest point of compression of the monolayer, the cross-sectional area of the phospholipid can be calculated. The detailed compression curve can give information on the relative influence of the head group and the fatty acid tails (92, 93).

2.2.1.2 Bilayers and Liposomes

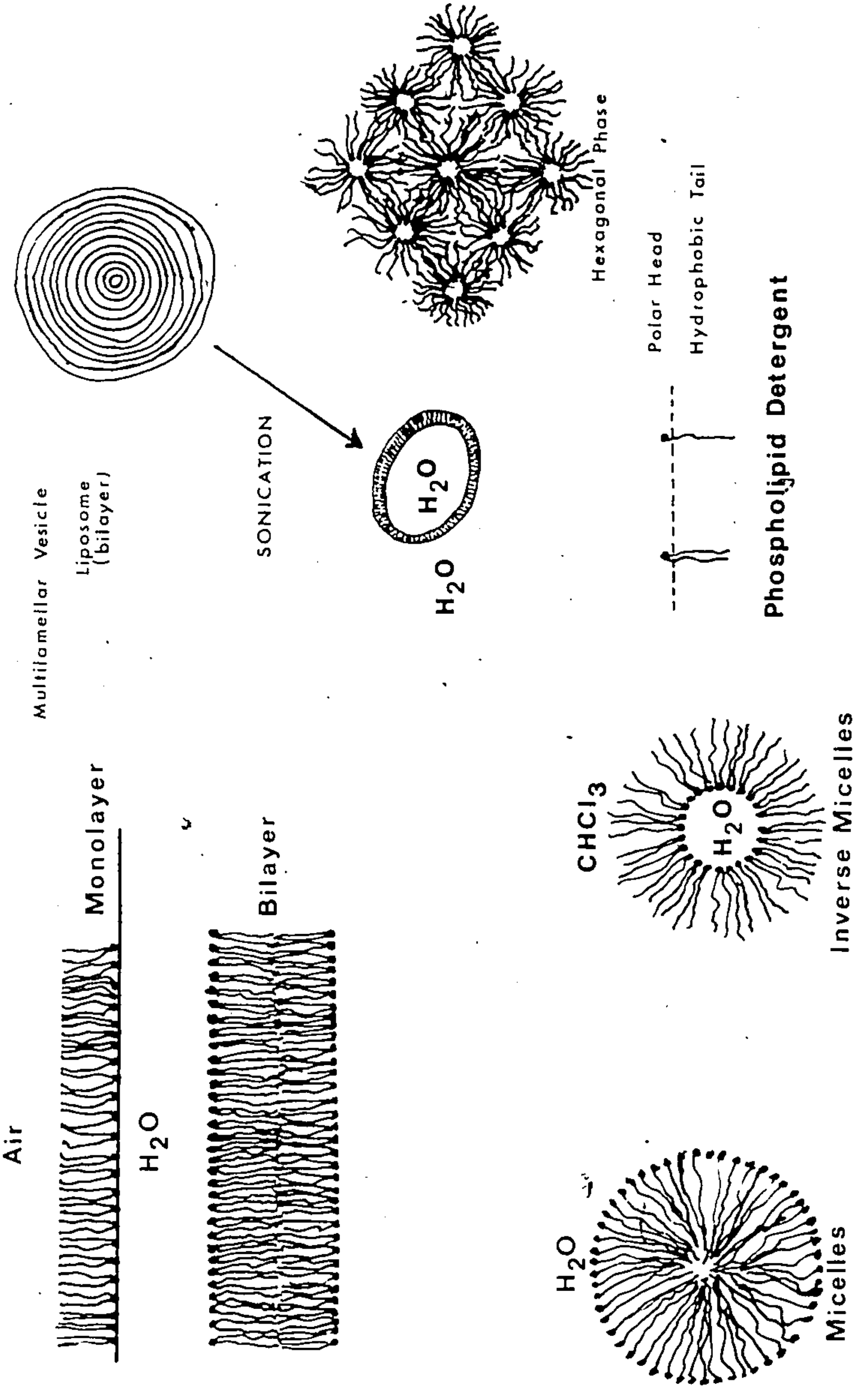
The most thermodynamically stable aggregate of lecithin is the bilayer (94, 95, 137). The phospholipids in this structure form a two-dimensional barrier to water with the polar head groups projecting into the aqueous medium and the hydrophobic chains projecting away from the water into the interior of the bilayer. This very ordered two dimensional crystal has a great tendency for spontaneous formation as judged by the enthalpy (ΔH) for bilayer formation from aqueous solution of -18 kcal/moles. It should be noted also that these bilayers will form only if the temperature is such that the phospholipid is in the fluid state. Bilayers can be seen as a 75 angstrom wide double track in EM on heavy metal staining of both biological membranes and phospholipid dispersions (96).

Bilayers of lecithin associate into large structures consisting of a series of concentric hollow spheres of bilayers, layered one on the other, much like an onion. This structure is called a multilamellar vesicle (MLV) or liposome (95) (Fig. 22). Each layer is formed of a bilayer, and between each layer is aqueous medium. If an aqueous dispersion of MLV is subjected to ultrasonic vibration the liposomes will be disrupted and fragmented to smaller unilamellar structures called vesicles (Fig. 22).

2.2.1.3 Model Membranes

The study of biomembranes is rendered easier when the components are separated and investigated individually. Though the properties of simple membranes are somewhat different to that of biological membranes, the results can be used to understand the behaviour found in more complex membranes. One of the most commonly studied phospholipids is dipalmitoylphosphatidyl choline (DPPC). PC is the most abundant phospholipid in most biomembranes and C16 fatty acids are usually present in natural membranes. DPPC is the major phospholipid of lung surfactant. DPPC is easily synthesized and is commercially available at a reasonable cost.

Figure 22: Mesomorphs formed by amphipaths in aqueous and non aqueous media.


Air
Monolayer
H_2O
Bilayer
Multilamellar Vesicle
Liposome (bilayer)
SONICATION
H_2O
H_2O
Hexagonal Phase
Polar Head
Hydrophobic Tail
Phospholipid Detergent
H_2O
Micelles
$CHCl_3$
H_2O
Inverse Micelles

2.2.2 Membrane Fluidity

Membrane fluidity can be defined in terms of viscosity in the following simple relationship (97):

$$\text{Fluidity} = \phi = \frac{1}{\text{viscosity}} = \frac{1}{n}$$

Because the conventional methods for the determination of viscosity measure the bulk properties of the liquid, and because it is the mobility of the individual lipids in the bilayer which is the area of interest in membrane fluidity, special methods are needed to examine membrane fluidity. Lipids in a bilayer are stacked parallel to one another and nearly perpendicular to the plane of the bilayer, forming a two dimensional crystal. The mobility of the lipids in a membrane can be considered in terms of 4 components of motion of the lipid:

1. Vibrational motion of the fatty acid chains of the phospholipids
2. Translational motion of the lipid along the plane of the bilayer.
3. Rotational motion of the lipid along the long axis of the lipid ie motion similar to the motion of a top.
4. Flip-flop motion of the lipid: movement from one side of the membrane to the other side.

The vibrational motion of the fatty acid is defined as the rate of exchange of <u>trans</u> and <u>gauche</u> conformers and is often measured as the relative amount of these two conformers at any time (98). This property can be measured by deuterium NMR and FTIR spectroscopy. Deuterium NMR experiments on aqueous dispersions of perdeuterated DPPC at 0°C have shown the fatty acid chains to be highly ordered and immobilized in a gel like state (Fig. 23) with the methylenes in an all *trans* conformation (99, 100). A gradual increase in motion is observed with increasing temperature. At the transition temperature the motion increases rapidly indicating the formation of *gauche* conformers. Mantsch (101, 102, 103) has confirmed these results with FTIR. They found that in the gel state, up to the transition temperature, all the methylene groups of the fatty acid chains are in the *trans* conformer. However, in the liquid-crystalline phase, there is an increase in the average number of gauche conformers per acyl chain as the temperature is raised (99).

The lateral diffusion rate of phospholipids along the two dimensional plane of the bilayer was measured by ESR and NMR spectroscopy. Using spin labeled lecithin (labeled in the head group) Kornberg (104) found the jump rate of a phospholipid from one site in the bilayer to another site to be greater than 3000 times per second at 25 °C. Several authors (105, 106, 107, 108) using different ESR methods (109) have found the diffusion rate of phospholipids in the bilayer and in the liquid crystalline state to be from 1 X 10^{-8} to 5 X 10^{-7} cm^{2}/sec. This implies that a phospholipid can travel thousands of molecular diameters a second; however the presence of proteins and cholesterol are known to slow down translational motion (110, 106). Lateral diffusion can give rise to phase separation in biological membranes and this in turn to functional heterogeneity along the surface of the bilayer.

Rotational motion along the long axis of the phospholipids in a bilayer also has been measured by ESR and NMR and has been found to be temperature and phase dependant. Results have shown the lipids rotate very rapidly in the fluid state (109).

Finally, flip flop motion has been found to be extremely slow in comparison to the other modes of motion. Single bilayer vesicles of lecithin with spin labelled lecithin only on one side have exchange rates slower than 2 X 10^{-5} times/sec (104). This is not surprising as studies of biological membranes have shown that the lipid of these to be highly asymmetric across the membrane (111).

It seems reasonable to assume that an increase in fluidity arising from various modes of motion of the lipids would cause the bilayer to expand. The expansion of sealed vesicles upon heating can be measured by the amount of light scattering in the visible range (650 nm) (112). This expansion or lowering of molar volume is quantified by the following equation:

$$\phi = 1/n = B(V-V_o)/V_o \qquad (97)$$

where Vo is the molar volume of the liquid just above its melting point, V is the volume of the liquid at the measured temperature and B is a constant to correct for the excess molar volume and for the spatial orientational features of a bilayer (97). Fluidity measurements using fluorescent reporter probes imbedded in membranes are actually measuring the degree of expansion of a bilayer (153). The steady state anisotropy or polarization is a measure of of the angular rotation of the probe which depends on the 'space' between the lipid molecules (113).

There is a close dependence of fluidity of biomembrane on the molecular structure of phospholipids (89). Organisms alter the molecular composition of these lipids to compensate for changes in their environment (83). The length and degree of unsaturation of fatty acid side chains as well as the nature of the polar head of a lipid has a profound effect on the fluidity of the membrane. These aspects will be discussed next under the heading of lipid thermotropic effects and are the immediately following topics.

2.2.3 Lipid Thermotropic Effects

2.2.3.1 The Gel State

At room temperature, aqueous dispersions of saturated derivatives of PC [di-16:0, di-18:0, di-20:0 etc] exist in a gel (Fig. 23) like state where van der Waals forces between the methylene groups of the fatty acid side chains maintain a crystal structure. As mentioned above, the methylene groups are held in an all trans conformation (see membrane fluidity section) with the fatty acids tilted slightly out of perpendicular to the plane of the bilayer (30°) (114). This is thought to allow for maximal separation of the hydrostatic repulsive forces of the head group of PC (87), while maintaining the tight orthorhombic packing of the fatty acid chains (Fig. 24) (102). There is also some torsional motion, which increases with increasing temperature of the bilayers (102).

2.2.3.2 The Pretransition (Tp)

As the temperature is increased there is an increase in motion (tortional) of the lipid chains and a rearrangement of the orthorhombic or distorted hexagonal packing of the fatty acids to hexagonal packing above Tp (114, 102) (Fig. 24), with a corresponding slight straightening up of the chains to less than 30° from perpendicular to the plane of the bilayer (Fig. 23) (114), an increase in the hydration of the head group (87) and the appearance of a periodic undulation or rippling along the surface of the bilayer, which can be distinctly seen, by freeze etch electron microscopy, as furrow like structures on the surface of the bilayer (167), and has been detected and the periodicity measured by X-ray diffraction (115, 114). This low heat requiring transition was first seen by calorimetry and is generally referred to as pretransition (Tp) (2).

2.2.3.3 The Main Transition (Tm)

As the temperature is further raised the motion of the chains increases to a point where the van der Waals bonds break and the fatty acids become fluidised. The temperature and the energy requirement of this melt was first measured by calorimetry and was found to be dependant on the length of the fatty acid chains of the phospholipids (2), as well as the polar head (2), where the temperature, enthalpy and entropy of transition increases nearly linearly with increasing chain length of the fatty acids (Fig. 30). The increase in van der Waals forces between fatty acid chains has been calculated to be approximately 1.0 Kcal/methylene (2). Also the difference in the temperature of the pretransition and the main transition diminishes linearly with increasing chain length (117).

2.2.3.4 The Liquid Crystalline State

The liquid crystalline state or the fluid state is characterized by an increased motion of the fatty acid chains (99, 100), by translational motion of the lipids along the plane of the membrane (106, 109), rotational motion of the lipid perpendicular to the plane of the membrane (98) and finally in an overall expansion of the bilayer (118, 119) (Fig. 23). This motion is discussed in the previous section on membrane fluidity. Spontaneous formation of liposomes will occur only if the lipid aqueous mixture is above the lipids' transition temperature. This implies that membranes must be fluid in order to form these special stuctures.

2.2.3.5 The Subtransition (Ts)

In aqueous dispersions of DPPC, the gel to liquid-crystalline main transition at 41.5 °C and the pretransition at 35 °C are the only thermal transitions usually seen by calorimetry. However liposomes of DPPC maintained at 0-4 °C for several days, show a third broad transition at 18 °C. The quantity of heat absorbed by this transition increases with the period of incubation at 0-4 °C to a maximum value at several days. For DPPC, the maximum heat of transition of the subtransition so far reported (4.5 Kcal/mole) was three times as large as the pretransition (1.5 Kcal/mole) and one half as large as the main transition (9.4 Kcal/mole) (120). The transition on cooling cannot be detected by DSC. This type of behaviour is a typical kinetically controlled slow process leading to a more thermodynamically stable state. Recent infrared spectroscopy studies have shown that this pretransition is caused by the rearrangement of the 2-dimensional packing of the acyl chains of DPPC (143) and possibly a change in the orientation and hydration of the head group (184, 185).

Figure 23: Thermotropic behaviour of saturated lecithins

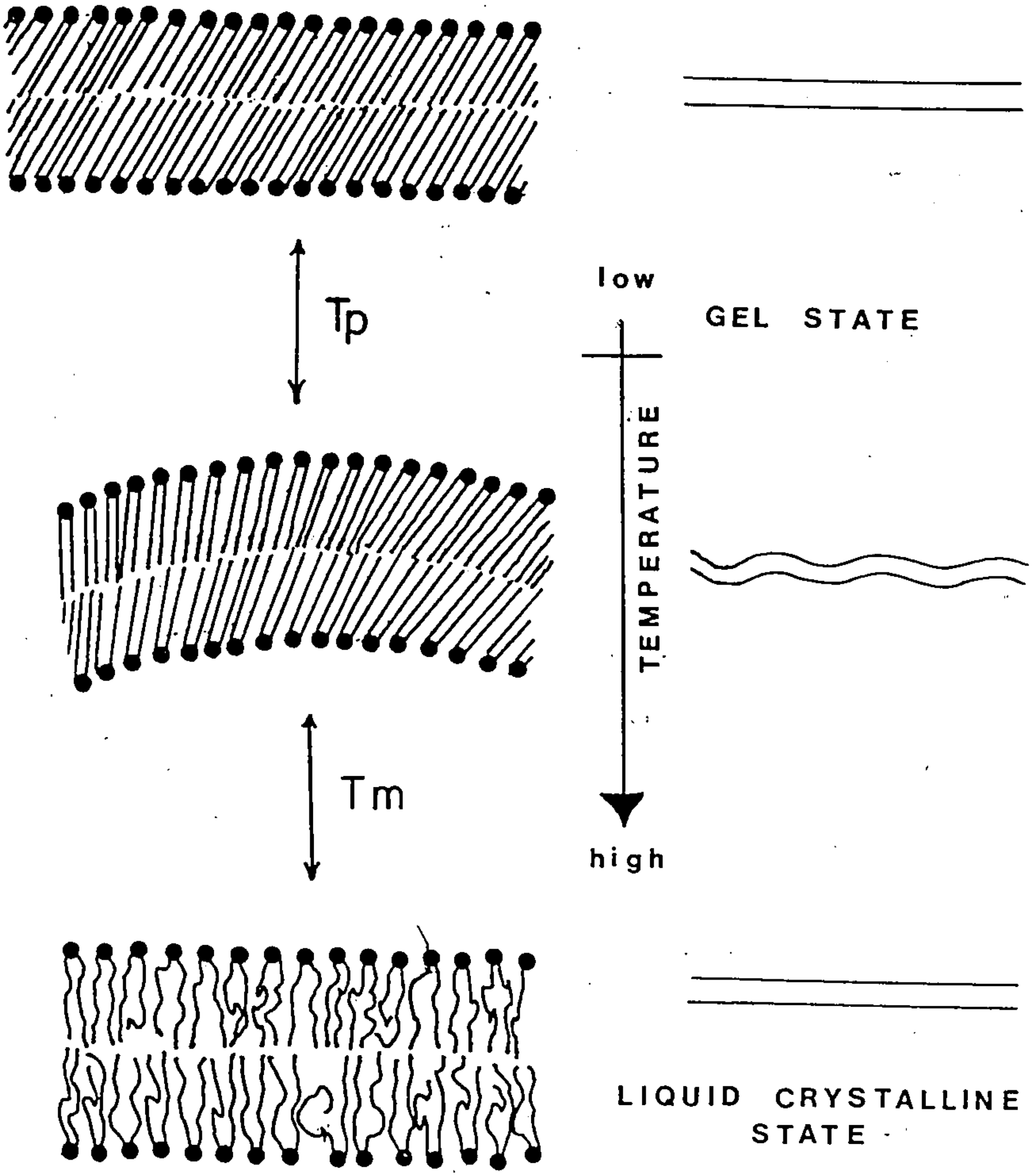
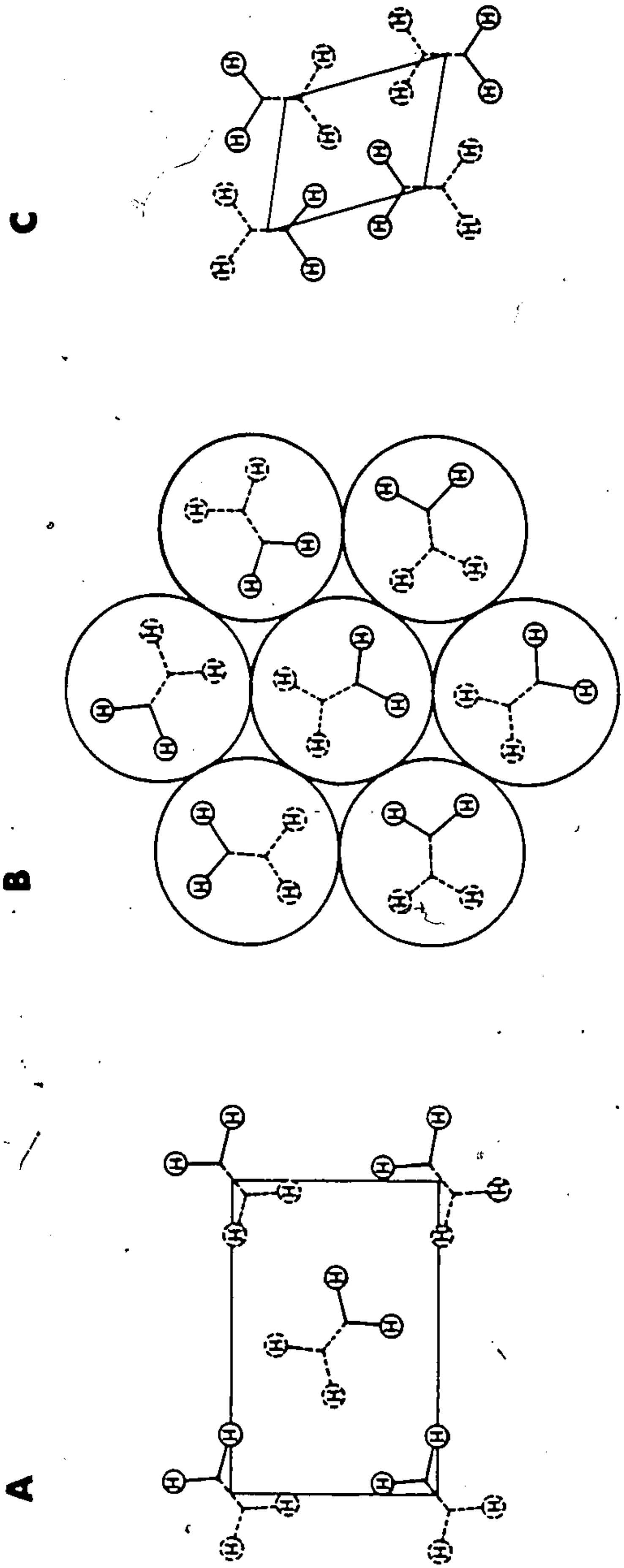

Tp
low
GEL STATE
TEMPERATURE
Tm
high
LIQUID CRYSTALLINE STATE

Figure 24: Crystal packing of acyl chains of phospholipids in the bilayer: A) orthorhombic, B) hexagonal and C) monoclinic.


A
B
C
H

2.2.4 Hexagonal Phase versus Bilayer

PE behaves quite differently than PC (122, 123, 124). Saturated derivatives of PE form bilayer structures but these have gel to liquid transitions 20 to 25 °C higher than the corresponding PC. At higher temperatures the fatty acid part of the molecules swing apart and the liposomes are transformed into cylinders stacked parallel on top of each other. Each cylinder is comprised of lipids with the polar heads pointing inward and the fatty acid tails are pointing outward. This structure does not hold very much water compared to the bilayer and is called hexagonal phase (Fig. 22), not to be confused with hexagonal packing (Fig. 24). Unsaturated PE like Egg PE or di-18:1 PE adopt the hexagonal phase at room temperature (122).

The different morphic behaviour of PE verses PC has been attributed to at least three different things:

1. The smaller head group of PE (125).
2. The difference in orientation of the two headgroups (126, 127, 128).
3. The ability of PE to form stronger polar head intermolecular associations with neighboring polar heads (129, 130, 131, 187).

Neutron diffraction studies of PC and PE have shown the head groups of both derivatives in both the gel and fluid states are nearly parallel to the plane of the bilayer.

(132). The evidence of a greater intermolecular association of PE head groups than of PC head groups via hydrogen bonding between the negative charged phosphate of one molecule and the positively charged ammonium group of a neighboring lipid molecule is strong:

1. The lower mobility of the head group of PE than PC as measured by NMR (133).
2. The greater difficulty in hydrating PE than PC (134).
3. PE packs tighter than PC in monolayer studies (87).
4. The higher transition temperature of PE (2).
5. The greater order of PE bilayers than PC bilayers (87).
6. Vesicles of PE interact with one another and fuse at a much more rapid rate than vesicles of PC (135, 130).
7. If the pH is raised the transition temperature of PE's drops to that of the corresponding PC and the vesicles of PE will no longer fuse (136) due to a deprotonation of the ammonium group. This indicates that a positive charge on the amine is necessary for intermolecular association of the polar head groups.

Examination of the transition temperatures of other phospholipids show that the transition temperature of DPPC at 41 °C is the lowest for all the different head groups. Thus DPPC(+-) has a transition temperature of 41 °C, DPPE(-, at high pH) at 41 °C, DPPG(-) at 41 °C (87). It has been suggested that the head groups of PC and these other phospholipids repulse each other and thus cause this low transition temperature (87). Other phospholipids, on the other hand, are thought to have interactive polar head groups and therefore higher transition temperatures. Thus DPPE (+-) has a transition temperature at 63 °C, DPPS(--+) at 55 °C, DPPS (-+) at 72 °C and DPPA (-) at 71 °C (87).

2.2.5 The Effect of Cholesterol

Cholesterol is an important component of animal plasma membranes and its effects on membranes has been studied extensively (141, 45).

Cholesterol has been reported to have both a fluidizing and condensing effect (141, 142, 42). How can this dual behaviour be explained?

First cholesterol interacts non-covalently with bilayers and disrupts the orthorhombic packing of the fatty acid chains (112). This restructuring of the chains accounts for the disappearance of the pretransition and the lowering of the cooperative unit of saturated lecithins. Also while the steroid nucleus immobilizes the first nine methylene carbons of the phospholipids, the remaining terminal fatty acid methylenes and the sterol side chain are very mobile. In addition, the head group and the <u>sn</u>-3 carbon of glycerol do not interact with cholesterol (193) while the fatty acid ester carbonyls hydrogen bond to the sterol hydroxyl (112). This explains the dual nature of cholesterol: its fluidizing and condensing powers.

Below 20 mole % cholesterol, two phases coexist and appear to be made up of pure DPPC bilayers and mixed bilayers of DPPC-cholesterol 8:1 (188) or 4:1 (189). At cholesterol levels greater than 20 mole % the separate domains no longer exist, the bilayer condenses and this stabilized structure behaves as a better permeability barrier to water and other metabolites (141). At 29 mole % cholesterol, there is another critical change in the structure. Despite the membrane stabilization effect cholesterol has very little influence on phospholipid diffusion rates (191). Addition of increasing quantities of cholesterol progressively abolishes the gel to liquid-crystalline transition with the appearance of an additional broad thermal transition at a slightly higher temperature (190). Finally, the bilayer cannot accomodate a greater cholesterol content than 50 mole percent before it is disrupted by the crystallisation of the cholesterol.

2.2.6 Fluid Mosaic Model

The fluid mosaic model, proposed by Singer in 1972 (144) is now generally accepted as being the prevailing model of the structure and function of biological membranes. This model proposes that the biological membrane of living cells is principally a bilayer structure with a 2 dimensional sea of lipids in which and on which floats a mosaic of proteins. Like any model, it has certain limitations. There is strong evidence that hexagonal phase may be required for fusion of membranes, a vital function in certain cellular processes like the secretion of hormones (145).

2.3 REVIEW OF THE PHYSICAL METHODS USED IN THE PRESENT STUDY

2.3.1 Calorimetry

Differential Scanning Calorimetry (DSC) is a technique in which the sample and reference are heated or cooled separately and the difference in heat required to maintain both at the same temperature is recorded against temperature (2). Thermal events such as phase transitions and changes in enthalpy may be accurately determined by DSC.

In DSC, aqueous dispersions of homogeneous lipids give very sharp endothermic gel to liquid-crystalline transition, an indication of a high degree of cooperativity between the lipids. A rough measure of the size of the cooperative unit of the monolayer is given by the expression $h/\triangle T$: where h = peak height and $\triangle T$ is the temperature range of the transition at a fixed height. The cooperative unit has also been calculated by other methods (117). The enthalpy of the process ($\triangle H$) is is given by the area under the transition peak while the change in entropy is derived from the following relation, for an adiabatic process (2):

$$S = \frac{\triangle H}{Tm(K)}$$

2.3.2 Fluorescence Studies

2.3.2.1 Fluorescence polarization

The degree of fluidity or the rigidity of bilayers may be measured by the technique of fluorescence polarization of a suitable fluorescent probe. The degree of polarization of emission using vertically polarized excitation source is defined as:

$$P = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}}$$

where $I_{//}$ and $I_{\perp}$ are the intensities of the components of the fluorescence measured parallel and perpendicular to the direction of the vertically polarized excitation beam (146). The fluorescence polarization is maximal when the fluorescent molecule is fixed in a given orientation (does not rotate during the lifetime of the excited states) and when the emission dipole is parallel to the absorption dipole. If this ideal situation can be achieved then the maximum value of P is 0.5. Therefore in a spherical liposome, if the fluorescent probe is completely immobilized then one expects to observe a value of 0.5. In practice the ideal situation is rarely achieved because the motion is never totally restricted and the absorption and emission dipoles are rarely parallel. The value of P is therefore always below 0.5. The polarization of a fluorescent probe such as DPH in the rigid gel state of DPPC is about 0.4. When the rigidity of the bilayer is diminished, as in the liquid-crystalline state, the fluorescent molecule has a greater freedom of motion resulting in a lower degree of polarization, value of 0.2-0.3. The transition temperature (Tm) is thus defined as the temperature at the mid-point in the change in P.

The fluorescence intensity of polycyclic aromatic molecules is dependent on the solvent, temperature, and phase (148, 147). For example, the fluorescent intensity (quantum yield) of unsubstituted aromatic molecules in aqueous media is generally low compared to that in non polar media, such as hydrocarbons. Therefore if such a membrane probe is dissolved in the external isotropic aqueous media its fluorescence will not interfere with the polarised fluorescence of the probes imbedded in the membranes. Of course, the partitioning equilibium of a membrane probe should lie heavily in favour of dissolution of the fluorescent molecule in the bilayer. The fluorescence intensity progessively diminishes with increasing temperature and drops rapidly at the gel to liquid-crystalline transition. This is because interactions with the lipid environment are increased resulting in an increase in the non-radiative deactivation of the excited state of the probe molecule. Hence fluorescence quenching is observed. The interference of other excited state quenching mechanisms such as oxygen quenching or due to excimer formation may be eliminated as follows: oxygen quenching is eliminated by the use of deoxygenated solutions while self-quenching (the formation of excimers) is minimized with dilute solutions of the probe in the bilayer. The high lipid to probe ratio (400:1) also lowers the disruptive effect of a foreign material in the bilayer. Because only small amounts of fluorescent probes are used, it is required that the fluorescent molecule has a high molar extinction coefficient.

A summary of the relevant properties of the fluorescent probes used in this study is given below. Four fluorescents probes were used: diphenylhexatriene, cis and trans parinaric acids and p-quaterphenyl.

2.3.3 Fluorescent Probes

2.3.3.1 Diphenylhexatriene

Diphenylhexatriene (DPH) (Fig. 25) an aromatic molecule used widely in many model and biological membrane studies (113, 149, 150, 151), is particularly suited for membrane studies. In particular, its extremely high molar extinction coefficient (85,000 units) ensures that very small amounts of this probe may be used which will not disrupt the membranes. The fluorescence of DPH can be quenched quite severely with oxygen but its quantum yield in deoxygenated solutions is very high (0.8). In addition, the hydrophobic and the linear nature of the excited molecule render this probe lipid soluble and hence its fluorescence polarization is dependent on the disorder of the bilayer (146). This probe reports from the hydrophobic region of the bilayer (152, 192) (Fig. 26).

2.3.3.2 Parinaric Acid (153, 154)

Parinaric acids are excellent fluorescent probes for studying membranes. They are unsaturated fatty acids with 4 conjugated double bonds. They exist in two forms: all <u>trans</u> (Fig. 25) and <u>cis</u> <u>trans</u> <u>trans</u> <u>cis</u> (Fig. 25). Because they are fatty acids they induce very little perturbation to the membrane and report from the polar regions of the bilayer (Fig. 26) (192). In addition, Sklar has reported that <u>trans</u> parinaric acid places itself in the ordered gel phase while cis-parinaric acid partitions into the liquid crystalline areas of the membranes.

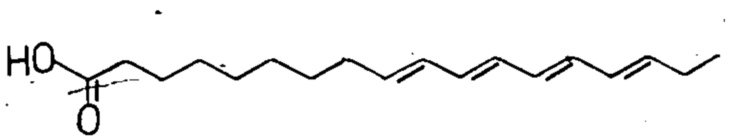
2.3.3.3 p-Quaterphenyl (QP)

This highly fluorescent molecule (Fig. 25) has not been used previously as a lipid probe. It has absorption and fluorescent properties desirable for a membrane probe: e.g. a high molar extinction coeficient (40,000 units); a high fluorescence quantum yield of (0.89) short singlet decay time (0.8 nsec) (147). The low solubility of p-quaterphenyl makes the handling of this substance difficult. As in DPH, QP is thought to report from the hydrophobic region of the bilayer (Fig. 26).

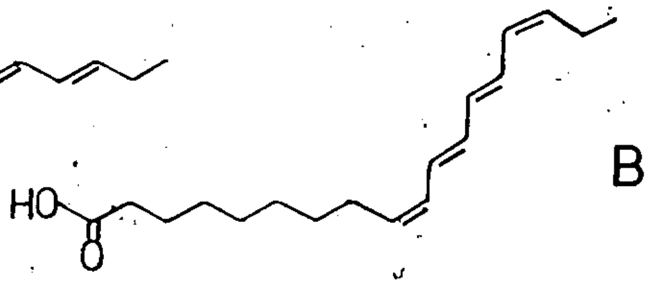
2.3.4 Electron Paramagnetic Resonance (162, 109)

McConnell and coworkers (155, 156, 157, 158) were the first to use stable nitroxide spin labels to study the physical properties of membranes. By comparing the spectra of spin probes in isotropic media and those intercalated in the bilayer, insight into the motion of the lipid molecules was obtained (157, 158, 168, 159). The fatty acid spin probes x-doxyl stearic acid proved to be very sensitive to motion along the long axis of the fatty acid chain because of the orientation of the unpaired pi electron orbital of the nitroxide group which lies parallel to the direction of the fatty acid chains. Through detailed spectral analysis, the rotational motion and the flexibility of the chains was analysed and was usually related in terms of the order parameter, S (160, 161). The spin probe N-tempo stearamide, which reports from the polar head region of the bilayer, has a more isotropic type motion because of the very high rotational rate of this probe around the short axis of the fatty acid chain. This rate of motion was related to the rotational correlation coefficient (162). Finally, the large nitroxide probes have been known to disrupt the bilayer structure and this has led to anomalies in the properties of the bilayer, e.g. low transition temperatures (169, 186). Two spin probes were used in the study of PSC: 5-doxyl stearic acid and N-tempo stearamide.

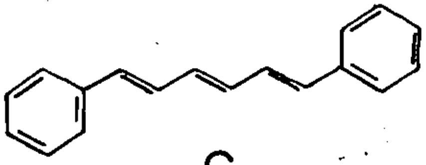
Figure 25: Structures of probes used: Fluorescent probes, A) <u>trans</u>-parinaric acid, B) <u>cis</u>-parinaric acid, C) diphenylhexatriene, D) quaterphenyl, Spin probes, E) 5-doxylstearic acid, F) N-tempo stearamide.



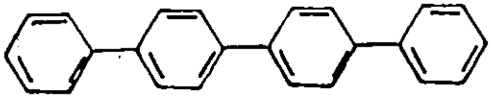
A



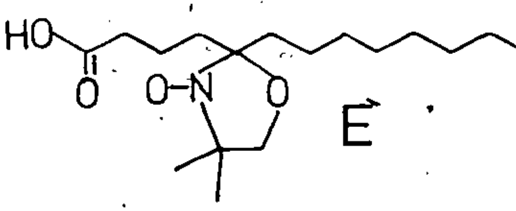
B



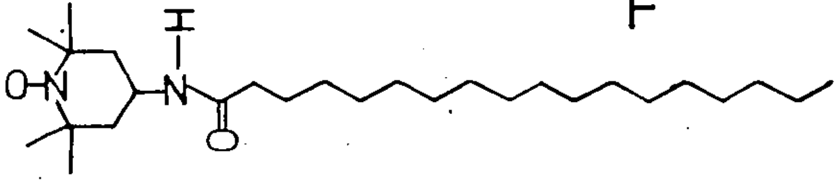
C



D

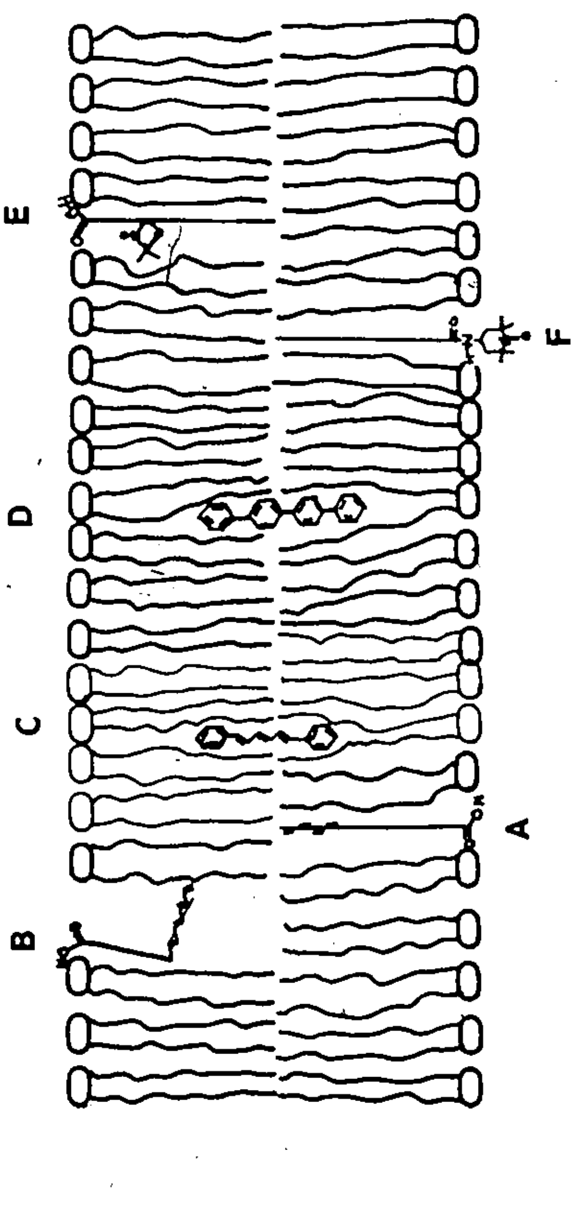


E



F

Figure 26: Probable location of probes in the bilayer: A) *trans*-parinaric acid, B) *cis*-parinaric acid,C) diphenylhexatriene, D) quaterphenyl, E) 5-doxyl stearic acid, F) N-tempostearamide.


E
D
C
B
F
A

2.3.5 Nuclear Magnetic Resonance

The NMR technique has been of great importance in the study of membranes. One great advantage is no extraneous probe is used, and therefore, the structure of the bilayer is not disrupted as in the techniques using probes. Phosphorus, proton, deuterium and carbon can be studied directly. Unfortunately, carbon and proton NMR give very little information on their environment: they show very little chemical shift anisotropy, and their relaxation times T1 and T2 are not significantly altered by small changes in motion. Deuterium and phosphorus on the other hand, have been used extensively in physical studies of membranes.

2.3.5.1 Deuterium NMR

Despite the relative insensitivity of deuterium NMR relative to proton NMR (9.65×10^{-3} times) the quadrupole moment of the deuterium nucleus (spin = 1) has made it particularly useful in the study of molecules in anisotropic media such as liquid crystals and solids (98). The observed quadrupole splitting of selectively deuterated lipids is influenced by local properties of conformation (order) and motion of the lipid molecules (132).

The application of an external magnetic field, Ho, influences the spatial orientation of the spins of the nucleus. The interactive energy Eq depends on the direction of the nuclear spins and on the distribution of local charges due to the C-D bond and these nuclear and electronic factors determine the orientation of the quadrupole tensor. The distribution of charges around the C-D bond produces an inhomogeneous magnetic field (electric field gradient tensor), with a symmetry axial to the direction of the C-D bond. Thus for a given EFG tensor angle, the interactive energy Eq has 2 possible values according to the allowed spins of deuterium (+1, -1). The two energy levels can be calculated or measured as the quadrupole splitting for fixed angles of single crystals and has been found to be -127 KHz for C-D bond perpendicular to Ho and 255 KHz for a parallel orientation using the static coupling constant 170 KHz (132). The deuterium spectra of liposomes, with deuterium labelled lipids appears as a powder spectrum due to the superpositioning of the quadrupole splitting of all the angles of the C-D bonds relative to Ho. The spectrum of the gel state (of liposomes) is very broad. However the spectrum of the liquid crystalline state, because of the rapid rotation of the lipid molecules about the long axis, is more narrow than the gel state spectrum because of the averaging of the signals about the axis of each lipid. In the spectrum of liposomes two maxima are seen; these correspond to 'effective angles of C-D' at 90° with respect of Ho and it is these maxima which are generally called "quadrupole splitting" as will be the case in subsequent discussion.

The deuterium spectra of lipids labeled in the head group cannot be analysed by single order parameters (166), as is commonly used for the spectra of phospholipid bilayers with deuterated acyl chains because there is a lack in knowledge of the behaviour of the head group (163). It is however increasingly apparent that more information is held in the deuterium NMR spectra than that obtainable from simple calculations of quadrapole splittings and signal halfwidths (164). More reliable deuterium spectra obtained from ultramodern high technology NMR spectrometers can give insight into aspects such as: the changes in the rates of motion of the lipids, in the distribution of the order parameters, in more accurate calculations of the average order parameters (100, 165), and in the fraction of the lipid in the gel or the fluid state (164). These various items may be calculated from the moments of the spectra according to the following equation:

$$M_n = \frac{\int_0^\infty x^n \, f(x)\, dx}{f(x)\, dx}$$

where f(x) is the experimental lineshape, $x = w_o - w$ and w_o is the angular frequency at the center of the spectrum. A more accurate measure of the quadrapole splitting is obtained from the calculation of the first moment of the NMR spectra [Dq=0.3849 M1, <u>vide</u> <u>infra</u>] (164). The fraction of the lipid in the gel state or the liquid-crystalline state at the temperatures just below and above the transition temperature can be calculated from the first, second and third moments by the method of Jarrell et al (164) using their equation 1.

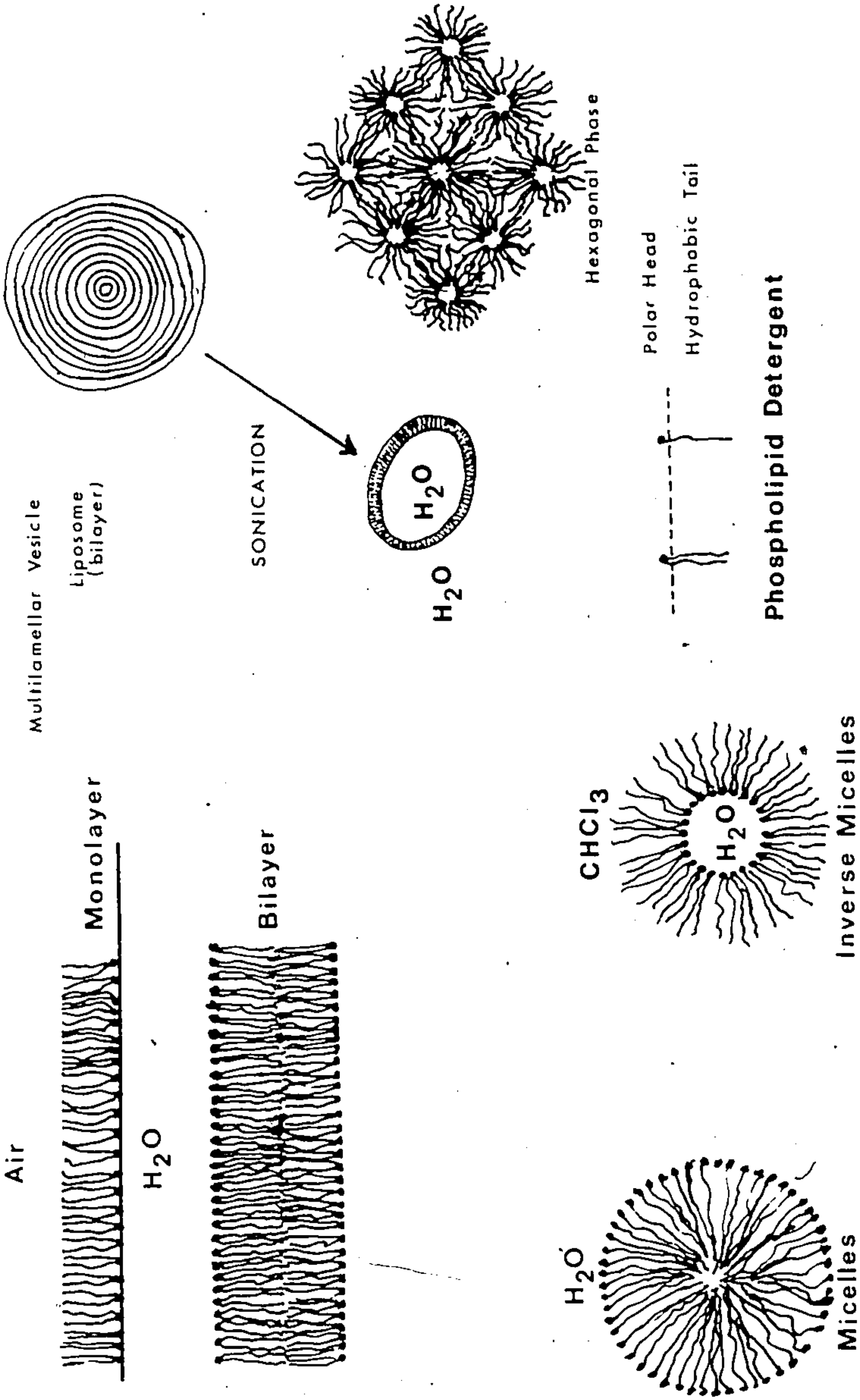
2.3.5.2 Phosphorus NMR (132)

^{31}P NMR is used to distinguish morphological phases and motional states of phospholipids. Unlike deuterium, phosphorus has a spin of 1/2 and its chemical shift is dependant on the orientation of the phosphorus nucleus in the magnetic field, because of the uneven distribution of the circulating electrons around the phosphorus nucleus. Phosphorus in isotropic solution will give rise to an average value, usually used as the reference chemical shift of zero. Different orientation of the phosphate group will result in the a shift upfield and downfield relative to the isotropic signal.

Phospholipids in a liposome are oriented with equal probablility over the surface of the sphere. A powder spectrum arises due to the superpositioning of the sub-spectra expected for each orientation of the phosphorus nucleus. Because the NMR signal is spacially oriented not all orientations have an equal contribution to the spectra and a characteristic powder spectra is obtained (Fig. 27). The orientation of the phospholipids in hexagonal phase is difference from that in liposomes and this results in that the spectra of hexagonal phase can be clearly distinguished from the spectra of bilayer phase (Fig 27).

Phosphorus NMR may be used in temperature dependence studies. In the case of liposomes, as the temperature is raised, the phosphorus spectrum narrows from the increase in motion and decrease in order of the phosphate. It is however, difficult to distinguish between these two parameters (166). The width of the spectra is nonetheless used to study the motion of lipids and is termed the extreme chemical shift (CSA) In CSA the difference between the upfield maximum and the lowfield minima is measured in Hz. The midpoint in sharp changes in this parameter is defined as the transition temperature (Tm).

Figure 27: Relationship between the ^{31}PNMR spectra and mesomorphic phases.


Air
Monolayer
H2O
Bilayer
H2O
Micelles
CHCl3
H2O
Inverse Micelles
Multilamellar Vesicle
Liposome (bilayer)
SONICATION
H2O
H2O
Polar Head
Hydrophobic Tail
Phospholipid Detergent
Hexagonal Phase

2.3.6 Fourier Transform Infrared Spectroscopy

Modern instrumental advances in IR spectroscopy, such as fourier transform spectra acquisition, has made it feasible to study model membranes due to the digitization, high resolution and a high signal to noise ratio of the spectra. Thus the frequency of a band may be accurately calculated from the centre of gravity, the band widths may be accurately determined at a fixed distance from the peak height and finally difference spectra can be used to measure previously undetectable thermotropic changes in the spectra.

The intensity, frequency and width of different bands have been used to characterize certain thermal behaviour such as the main transition temperature (Tm), the pretransition (Tp) and the nature of the gel and fluid states. The packing of hydrocarbon chains in a gel like phase has been determined by the splitting of the methylene scissoring band (1462 & 1472 cm^{-1}). In the case of DPPC in the gel phase below the pretransition temperature, the methylene scissoring band is split in two bands due to the "crystal field effect" that only occurs in an orthorhombic or monoclinic lattice (Fig. 24). On the other hand, the single band indicates a hexagonal or triclinic packing of the acyl chains and is found in the gel phase of DPPC above the pretransition temperature (Fig. 24). Other studies have confirmed that the packing of the acyl chains in gel phase at low temperatures is the tight orthorhombic subcell while at temperatures above the pretransition the chains are packed in a hexagonal manner (102)(Fig. 24). The pretransition temperature may be calculated from the methylene scissoring band (splitting) or from the changes in frequency and width of the ester carbonyl stretching band (1740 cm^{-1}). The main transition temperature can be accurately measured from several bands: methylene antisymmetric and symmetric stretching bands (2920, 2880 cm^{-1}) and the terminal methyl asymmetric and symmetric stretching band (2956, 2872 cm^{-1}) (103). These bands from the acyl chains show sharp changes in the intensity, frequency and bandwidth at the main transition temperature.

The strong phosphate bands (1220 and 1080 cm^{-1}) are rather insensitive to changes in temperature and phase, perhaps due to the anchoring effect of the complexed water. In addition, the head methyl groups of PC gives rise to a very weak band with minimal changes.

2.4 METHODS

2.4.1 Electron Microscopy

Freeze-fracture EM was carried out as described elsewhere (181) using a Polaron Type 7500 freeze-etch device equipped with an Edwards 12E6 coating unit and a Phillips Model 300 electron microscope.

2.4.2 Differential Scanning Calorimetry (2)

The differential calorimeter used was a Perkin-Elmer DSC-2 instrument with an operating temperature range between -60 °C and 400 °C. Solid samples (1-2 mg) were placed in sealable Dupont coated aluminum pans; water (2 to 10 μL) was added, and the mixture was stirred on a hot plate at a temperature 5 degrees higher than the expected transition temperature (Tm) of the lipid. With long chain saturated derivatives, addition of an extra aliquot of water (2 to 10 uL) was required to compensate for losses due to evaporation during heating at temperatures greater than 40°C. The pans were sealed by crimping the edges in a specially designed press and the samples were annealed by cycling their temperature above and below the transition temperature of the lipid and with vigorous shaking on a vortex mixer. The pan was then placed in the sample chamber of the DSC instrument and the sample was annealed further by heating and cooling it above and below the Tm until a reproducible DSC curve was obtained and no transition due to any unhydrated lipid was observed.

With non-solid lipids (e.g. dioleoyl phosphatidyl sulfocholine or egg PSC and DPPSC:cholesterol mixtures) chloroform solutions of the samples (2 mg/100 μl) were applied to the pans in small aliquots and brought to dryness under a gentle stream of nitrogen. Last traces of solvent were removed in a vacuum oven (0.05 mm of Hg) over KOH pellets at 40 °C for one half hour. These lipid films were then treated with water as above.

The melting behaviour of the dry crystalline PSC's, precipitated from acetone, were also measured by DSC. Samples were placed in the pans as described above, but the hydration and the annealing steps were omitted.

A heating rate of 2.5 °C per minute and a sensitivity of 0.5 to 2 mcal/sec² was used routinely. Enthalpies of transition were estimated from the areas of the transition peaks after calibration of the instrument with indium and quantitation of the phospholipid in the aluminium pans via fatty acid methyl esters analysis by GC as described in section 1.3.3.4. Areas of peaks were determined by cutting the peaks off the photocopied chart paper and weighing them. The chart speed was increased to 160 inches/min in order to increase the size of the peak and thereby lower the error in determination of their weight.

2.4.3 Fluorescent Spectroscopy

Fluorescence measurements were made with a Perkin Elmer spectrofluorimeter, model MPF-44A equipped with a Perkin-Elmer correction spectral unit. The fluorescent probes; (diphenylhexatriene (DPH), cis and trans parinaric acids or p-quaterphenyl) were incorporated into the lipids, in a probe to lipid mole ratio of approximately 1 to 400 as follows: a chloroform solution (ca. 1 mL) containing 0.6-0.8 mg (1 μmole) of phospholipid (PC or PSC) and 0.54 μg (2.3 nmoles) of DPH, or 0.64 μg (2.3 nmoles) of *cis* or *trans* parinaric acid, or 0.7 μg (2.3 nmoles) of quaterphenyl was brought to dryness in a 10 mL test tube under a steam of nitrogen. The residue was dried in a desiccator *in vacuo* (1 mm Hg), over phosphorus pentoxide, for thirty minutes. It was dispersed in 2.5 mL of 0.02 M sodium phosphate buffer (pH 7) in 0.1 M NaCl by warming above the transition temperature for 30 minutes with intermittent vigorous vortexing. The suspension was then transferred to a 3 mL clean stoppered cuvette containing a small magnetic stirring bar. The suspension was then deoxygenated with a stream of nitrogen for 8 minutes. The cuvette was sealed immediately with a tight fitting teflon cap and placed in the fluorimeter sample holder equipped with a small mini magnetic stirrer. Fluorescence measurements were made using the following wavelengths for excitation and emission: 360 nm and 430 nm, respectively, for DPH; 305 nm and 410 nm respectively, for *trans*-parinaric acid; and 310 nm and 415 nm, respectively, for *cis*-parinaric acid; and 295 nm and 365 nm, respectively for p-quaterphenyl. The temperature of the stirred suspensions was monitored with a thermocouple immersed in the suspension in a corner of the cuvette away from the paths of the excitation beam and the measured emission beam. Samples of phosphatidyl sulfocholine and phosphatidyl choline with identical fatty acid chains were placed in the same thermostated block and measured at the same time in order to minimize errors that might occur between runs. The degree of polarization was obtained by measuring the fluorescence intensities of both parallel (VV) and the perpendicular (VH) to the vertical polarized excitation beam using polaroid polarizing filters (type HNP'B). The different sensitivity of the fluorimeter for different polarization directions was corrected. The correction factor, G,

$$G = HH / HV$$

where HH indicates the fluorescence intensity when the direction of both the excitation and the emission beams polarization filters were horizontal and HV refers to the fluorescence intensity where the excitation beam filter was placed horizontally and the emission beam filter was placed vertically. Fluorescence polarization was calculated using the following formula:

$$P' = \frac{I_{VV} - I_{VH} \times (G)}{I_{VV} + I_{VH} \times (G)}$$

2.4.4 Electron Paramagnetic Resonance

EPR spectra were obtained in the laboratory of Dr. N.Z. Stanacev, Dept. of Clinical Biology of the University of Toronto with a Varian E-4 instrument operating at 9.5 GHz, equipped with a nitrogen gas flow variable temperature control unit. The sample was prepared as follows: a chloroform solution containing 20 mg (27 µmoles) of phosphatidyl choline or phosphatidyl sulfocholine and 75 nmoles of 5-doxyl stearic acid or N-tempostearamide spin probe was brought to dryness under a stream of nitrogen, in a 15 mL centrifuge tube. The residue was dried in *in vacuo*, dispersed in 0.08 ml of 0.024 M sodium phosphate buffer (pH 7.0) in 0.1 M NaCl, as described above. The samples were then transferred to a 50 µL disposable glass capillaries which were then sealed at one end. The instrument settings used routinely were: microwave power, 10 mW; modulation amplitude, 1.25 Tesla; scan range, 100 Tesla; and filter time constant, 0.1 seconds for 4 minute scans. Spectra were obtained at various temperatures and the order parameter (S) was calculated for 5-doxyl stearic acid from the following equation (168, 169):

$$S = \frac{T_{\|} - T_{\perp}}{27.42} \times \frac{15.18}{\frac{2T_{\perp} + T_{\|}}{3}}$$

For the spin probe N-tempo stearamide the rotational correlation time was calculated according to Schreier _et al_. (170). The order parameter and the log of the rotational correlation time were plotted against temperature.

2.4.5 Nuclear Magnetic Resonance (Broad Band)

2.4.5.1 Sample Preparation

One hundred mg of dipalmitoylphosphatidyl-S-[^{2}H]-methyl-S-methyl-mercapto ethanol (d-3-DPPSC) and 200 mg of deuterium depleted water (Aldrich) in a 1X5 cm quartz NMR tube were agitated on a Vortex mixer at 50 °C in order to make multilamellar vesicles (MLV).

2.4.5.2 Deuterium NMR

Deuterium NMR spectra were measured on a Bruker CXP-300 spectrometer operating at 46.1 mHz with the probe built by Dr. I.C.P. Smith's group (National Research Council, Ottawa, Canada.). Spectra were obtained by the quadrapole echo technique (98). The pulse spacing was 200 μsec; the recycle time was 1 second; the 90° pulses were set for 4.5 usec and 1000 acquisition were obtained per spectra. The delay between the second 90° pulse and the echo aquisition was set at 30 usec to obtain the maximum echo amplitude before Fourier transformation. The moments of the spectra were calculated on the ASPECT computer by Dr. Byrd of National Research Council of Canada as described elsewhere (166).

2.4.5.3 Phosphorus NMR

Phosphorus NMR spectra were measured on a Bruker CXP-300 spectrameter operating at 121.46 MHz. The spectra were obtained using 90° pulses of 8 μsec with 1 second recycle times and 200-500 acquisitions. The spectra were proton decoupled using a high power radio frequency signal decoupler.

2.4.6 Fourier Transform Infrared Spectroscopy

2.4.6.1 Sample preparation

Aqueous dispersions of DMPSC, DPPSC and DSPSC were prepared by adding 20 mg of deuterium oxide to 2 mg of dry crystalline PSC. The samples were heated in a water bath at a temperature five degrees above the corresponding gel to liquid-crystalline transition temperature and cooled to room temperature. This heating-cooling cycle was repeated three times. The dispersions were transferred to an infrared cell with calcium fluoride or barium fluoride windows and a variable thickness of 6, 12 or 25 μm.

2.4.6.2 Measurement of Spectra

The spectra were recorded on a Digilab FTS-11 Fourier transform infrared spectrometer and a high sensitivity cadmium telluride detector with 600 interferograms collected per spectrum. The spectra were computed from the interferograms as was described elsewhere (102), to a final resolution of 2 cm^{-1}. Temperature control was achieved by circulating a themostated mixture of methanol/water (1:1, v/v) through the hollow cell mount. The temperature was monitored with a copper-constantan thermocouple and was held constant within ±0.05 °C at each temperature for 16 minutes before measurement of each spectrum. The temperature was increased in increments of 2 C° corresponding to an overall heating rate (assuming noninterrupted heating as in DSC) of 0.125 C°/minute. The entire process, including the collection of the interferograms, the computing of the spectra, the incrementing and recording of the temperature, the temperature stabilisation period and the cycling process was completely under the control of the spectrometer's computer (101).

The subtransition experiment on DPPSC was measured with a Digilab FTS-15 Fourier transform instrument equipped with a wide range MCT detector, in a 50 μm thick cell fitted with barium fluoride windows. One thousand scans were accumulated for each spectrum.

2.4.6.3 Analysis of Spectra

The spectra were also analysed with the aid of the computer as described elsewhere (103). Thus, the frequency, width and intensity of the bands were calculated as discussed elsewhere (103). The maximum resolution capacity for the detection of changes in frequency and bandwidth was 0.1 cm^{-1}.

Finally, the difference spectra (Adiff) were also calculated by substracting the lower temperature spectra (AT^{-1}) from the next higher temperature spectra (AT) and normalized with respect to the temperature interval (ΔT):

$$A_{diff} = \frac{AT^{-1} - AT}{\Delta T}$$

2.4.7 Monolayer Studies

The monolayer studies were carried out on a Cahn surface tension (Wilhelmy plate method) device model 2000RT, equipped with a Cahn electrobalance model G5C, and a Easterling-Angus 2-dimensional recorder model 2400 A3 whose X axis was connected to the film area output and the Y axis was connected to the electrobalance. The measurements were carried out as described in the devices' instruction manual, using double distilled water in the trough which was thermostated at 25 °C. The minimal cross sectional area measurement of stearic acid was used as an independent check of the calibration of the device.

2.5 RESULTS OF PHYSICAL STUDIES

2.5.1 Electron Microscopy

Freeze-fracture EM of liposomes of egg-PSC (Fig. 28) showed typical multibilayer structures which were very similar to those observed with egg-PC (Fig. 28). Freeze-fracture EM of 16:0-PSC and 14:0 PSC aqueous dispersions showed replicas that were essentially the same as those of the corresponding PC analogues. Some differences, however, were evident in the surface features of the liposomes, depending on the quenching temperature employed. Thus, the saturated PSCs showed little or no surface features while the PCs often displayed a periodic rippled surface structure (167).

2.5.2 Differential Scanning Calorimetry

2.5.2.1 Saturated PSCs

The DSC scans of aqueous dispersions of the saturated synthetic PSCs (di-14:0, di-16:0, di-18:0) showed sharp main transitions (Tm) (Fig. 29), 2-4 °C higher than the transitions of the corresponding PCs (Table 5). Di-16:0 and di-18:0 PSC's did not show the characteristic pretransition found with the corresponding PC analogues. On the other hand di-14:0 PSC had a small and sharp pretransition 2.5 °C below its main transition, while the analogous di-14:0-PC has a small and broader pretransition, 7 °C below its main transition (Table 5). The change in Tm was also proportional to the fatty acid chain length in these saturated derivatives (Fig. 30).

2.5.2.2 Unsaturated PSCs

The unsaturated di-18:1-PSC however had a lower Tm (2.5 °C) than that of corresponding di-18:1-PC and had a pretransition 5 °C lower than its Tm; in contrast the ammonium analog di-18:1-PC had no pretransition (Table 5). Egg PSC had one broad main transition (-20 to -10 °C) which does not differ from that of egg PC. The fact that egg PSC is a mixture of molecular species may mask the effect of the head group.

2.5.2.3 Enthalpy and Entropy

The change in enthalpy, calculated from the area under the main transitions showed that the saturated PSCs as well as 18:1 PSC had almost identical $\triangle$ H's, within experimental error, to those reported for the corresponding PCs (Table 6). The changes in entropy for the gel to liquid-crystalline transition calculated for the PSCs were also identical to those reported for the corresponding PCs (Table 6), within experimental error.

2.5.2.4 Effect of Cholesterol

Addition of increasing proportions of cholesterol to DPPSC resulted in the gradual abolition of the transition seen at 45°C with the appearance of a very broad transtion centered at approximately 60 °C (Fig. 31). The transition from the gel to liquid-crystalline state at 45 °C completely disappeared on addition of cholesterol at a mole ratio of 1:1. The behaviour parallels that found in DPPC (2) except that disappearance of the pretransition at a cholesterol to DPPC mole ratio of 1:9 was not observed, since no pretransition occurs in DPPSC.

Figure 28: Freeze fracture EM of liposomes of: A) egg-PC, and B) egg-PSC.

Figure 29: Differential scanning calorimetry of PSC liposomes.

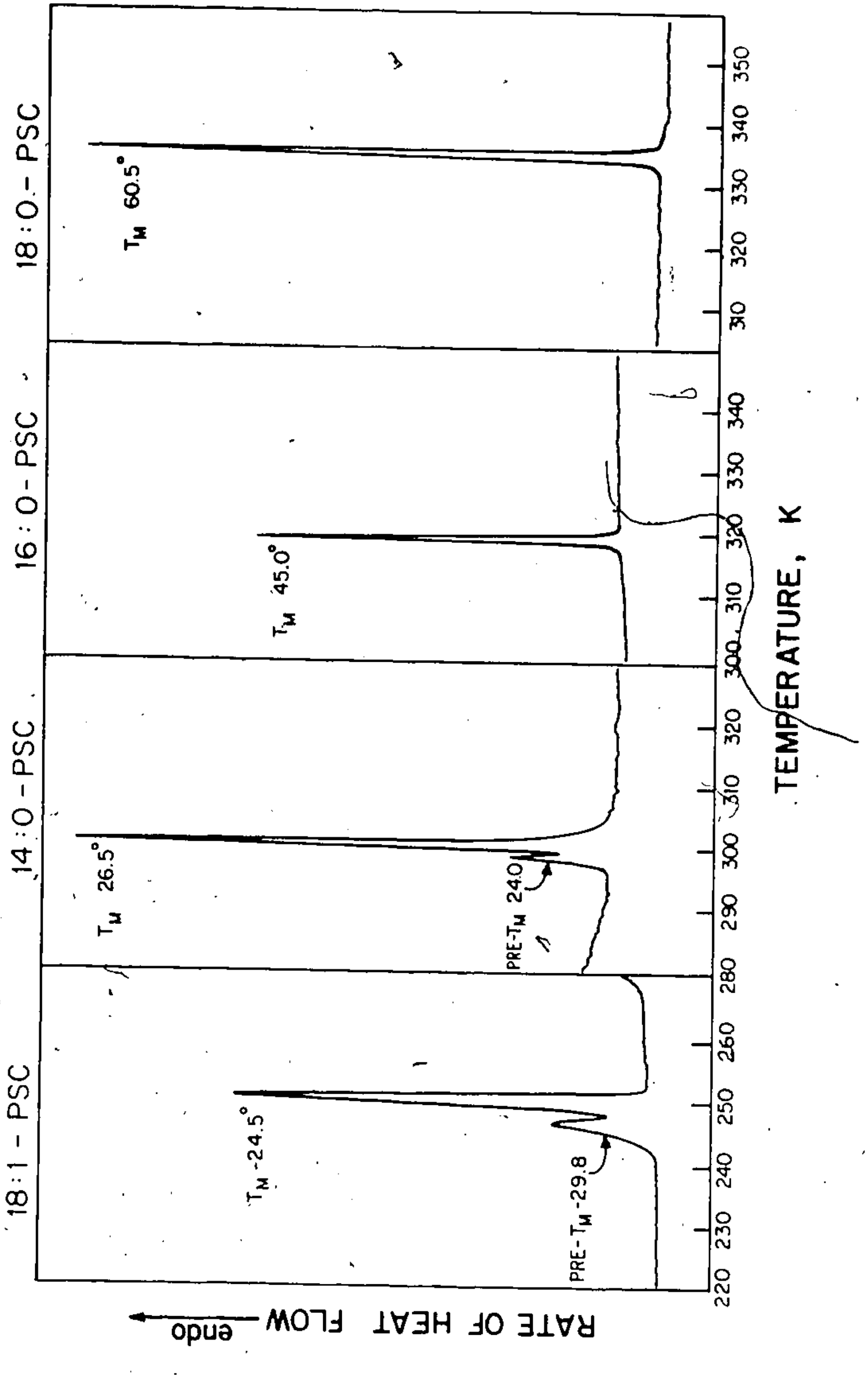

18:1 - PSC
T_M -24.5°
PRE-T_M -29.8
14:0 - PSC
T_M 26.5°
PRE-T_M 24.0
16:0 - PSC
T_M 45.0°
18:0 - PSC
T_M 60.5°
220
230
240
250
260
280
290
300
310
320
300
310
320
330
340
310
320
330
340
350
TEMPERATURE, K
RATE OF HEAT FLOW
endo

Figure 30: Relationship between Tm and fatty acid chain length of PC and PSC

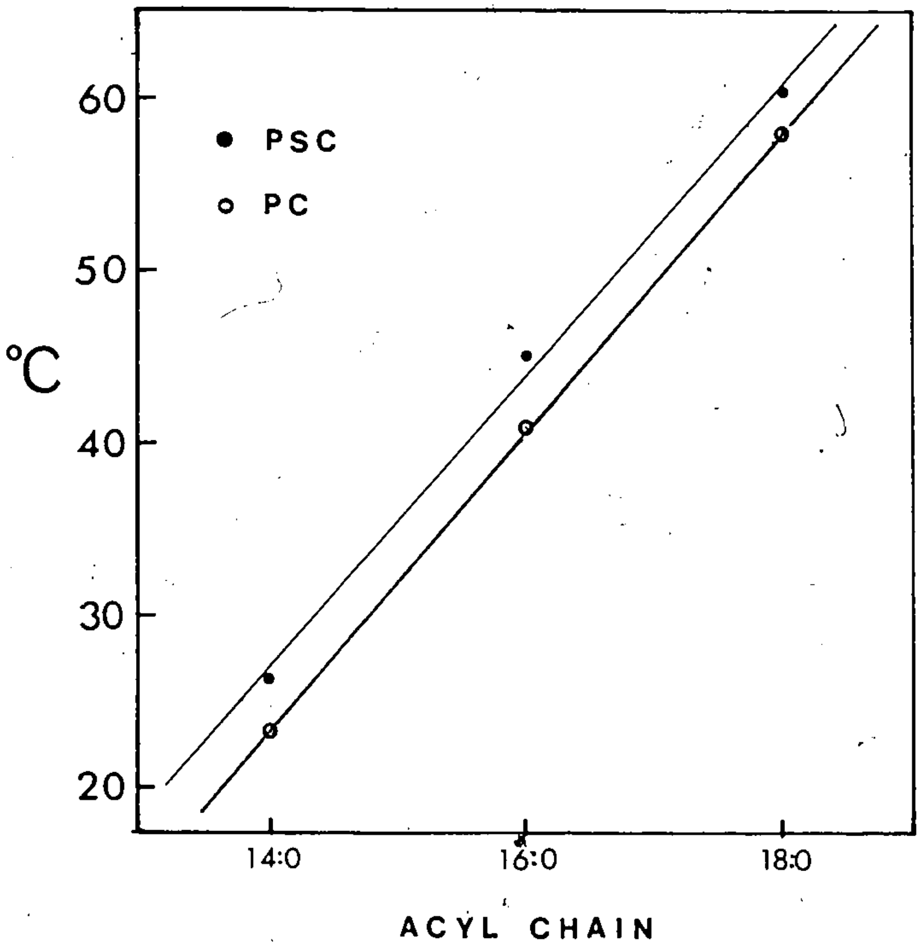

60
50
40
30
20
°C
● PSC
○ PC
14:0
16:0
18:0
ACYL CHAIN

Figure 31: Effect of cholesterol on the gel-liquid crystalline transition of 16:0-PSC.

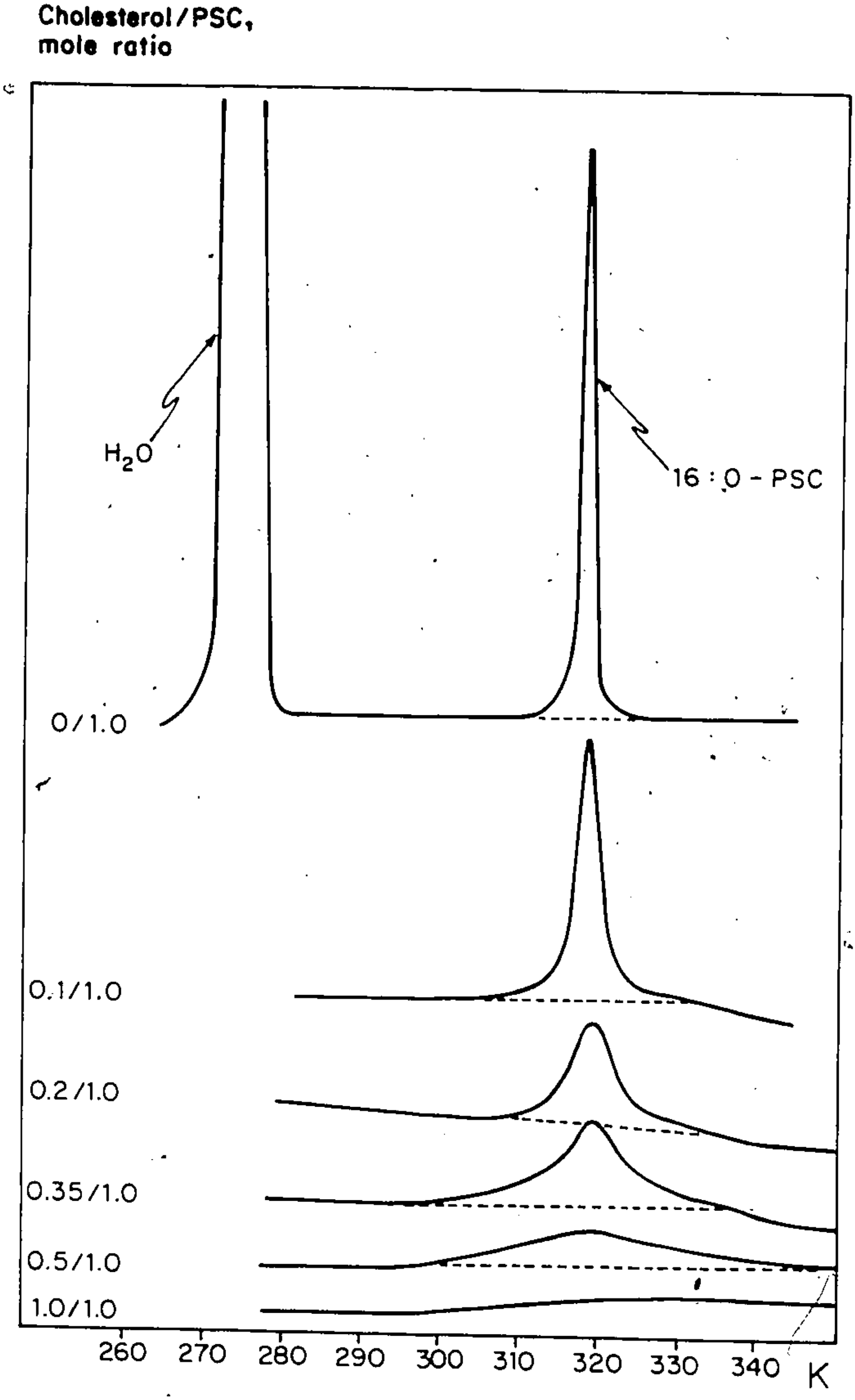

Cholesterol/PSC,
mole ratio
H2O
16:0-PSC
0/1.0
0.1/1.0
0.2/1.0
0.35/1.0
0.5/1.0
1.0/1.0
260
270
280
290
300
310
320
330
340
K

TABLE 5

Transition Temperature Data of PSCs and PCs as determined by various techniques

Acyl chain	Transition temperature [a], °C					
	DSC		Fluorimetry [b]		EPR [c]	
	PC	PSC	PC	PSC	PC	PSC
18:1	-22.0	-24.5 (-29.8)	-	-	-	-
14:0	23.0 (16.0)	26.5 (24.0)	25.5	28.0	23.0 (18.0)	25.0 (19.0)
16:0	41.0 (35.0)	45.5	41.0	45.5	38.0 (31)	43.0
18:0	58.0 (57.0)	60.5	58.5	58.0	45.0 (41)	54.0

[a] Values in parentheses are pre-transition temperatures

[b] With diphenylhexatriene as probe.

[c] With 5-doxyl stearic acid as probe.

TABLE 6

Enthalpy and Entropy Data for Main Transition of PSCs and PCs

Acyl Chain	PSCs			PCs *		
	Tm, K	ΔH kcal.mol^{-1}	ΔS $\frac{\text{cal.}}{\text{mole.degree}}$	Tm, K	ΔH $\frac{\text{kcal}}{\text{mole}}$	ΔS $\frac{\text{cal}}{\text{mol.degree}}$
18:1	248.8	6.7	27	251.3	7.6	30
14:0	299.8	6.5 ± 0.5	22 ± 2	297.0	6.3, 6.8	21, 23
16:0	318.8	9.4 ± 0.5	30 ± 2	315.0	9.7, 8.6	31, 27
18:0	333.8	10.5 ± 0.5	32 ± 2	331.5	10.8, 12	33, 36

* Data given for 18:1-PC are from ref. (2) and for 14:0-, 16:0 18:0-PC are from refs. (117) and (2).

2.5.3 Steady State Fluorescence

Fluorescent measurements on dispersions of PSC and PC, with the probes diphenylhexatriene, cis and trans parinaric acids or p-quaterphenyl showed that the probe was imbedded in the lipid as evidenced by the high fluorescence intensity and the polarised fluorescence obtained. The intensity of fluorescence progressively diminished as the temperature was raised (change in quantum yield with temperature) and diminished suddenly at the transition temperature.

The temperature dependent behaviour of fluorescence polarization of DPH was measured with the three saturated synthetic PSCs and equimolar PC and PSC mixtures (Fig. 32). The fluorescence polarization in the gel state of all the saturated PSCs was nearly constant with a value between 0.39 to 0.40, close to but less than the theoretical value of 0.5. A similar behaviour was found for the corresponding PCs except in DPPC where there was a steady decline in the polarization in the gel state. The fluorescence polarization at temperatures below the pretransition was slightly higher for the PCs than for the PSCs and with a value of 0.42 (Fig. 32). The main transition was characterized, in both derivatives, by a sudden lowering of the fluorescence polarization from a value of 0.4 to 0.15. The transition temperatures, taken at the midpoint between the gel and fluid phases correspond very well with the values found in the calorimetry studies (Table 5). No definitive evidence of a pretransition for the PSCs was seen in fluorescence polarization or in the fluorescence intensity studies except that a slight drop in the fluorescence polarization of di-14:0 PSC (Fig. 32 A) was observed at a temperature which corresponds to the pretransition found by DSC for this derivative (Table 5). The change was only about 0.04 polarization units and thus may be indicative of a slight change in the membrane structure, but not of a major fluidization of the membrane. On the other hand a pretransition was observed by fluorescence polarization for the PCs. Thus, for di-14:0, di-16:0, and di-18:0 PCs the pretransition temperatures (Tp) were 6.5, 4.0, and 6.0 °C respectively below the main transition temperatures (Tm) (Table 5). In contrast, the fluorescence polarization of the fluid state for the various PCs and PSCs were approximately identical.

Aqueous dispersions of equimolar mixtures of PSC and PC with the same fatty acid chains were studied using DPH as the fluorescent probe, in order to determine whether the two lipids were miscible. All mixtures showed a high fluorescence polarization in the gel state (0.40) except for the di-14:0 mixture where a somewhat higher value (0.42) was seen at 10 °C. All mixtures showed no evidence of a pretransition except the di-14:0 mixture which gave a small change in the fluorescence polarization starting 5 degrees below the onset of the main transition (Fig. 32 D). The onset temperature of the Tm of the mixture corresponded well with the onset temperature of melting of pure PC derivative while the temperature of the termination of melting corresponded with the temperature of the end of melting of the pure PSC derivative (Fig. 32 D). The curves of the mixtures actually fall between the curves for the individual PC and PSC.

The results obtained with the parinaric acids showed additional differences between the two lipid analogues. The main transition temperature for DPPSC and DPPC as noted by polarization measurement were the same as found with the DPH probe or by calorimetry: i.e. 45 °C for DPPSC and 41 °C for DPPC (Fig. 33). The drop in fluorescence polarization accross the phase transition was not as great with the parinaric acids as with DPH ($\triangle$ P=0.1 with the parinaric acids and $\triangle$ P=0.35 with DPH). The difference between the fluorescence polarization in the gel phase of DPPC and DPPSC was always greater with parinaric acid than with DPH (Figs 33 and 32). Furthermore the fluorescence polarization of DPPC with the <u>trans</u>- parinaric acid probe was higher than that of DPPSC by 0.08 units in both the gel and fluid states. In contrast, with <u>cis</u>-parinaric acid probe, the polarization of the gel state for DPPSC was the same as the polarization of the fluid state of DPPC (Fig. 33). Also the onset of the transition observed with the cis-parinaric acid was about one degree lower than the onset with the <u>trans</u>

parinaric acid. The mid point of the transition is however at the same temperature for the two derivatives.

Quaterphenyl gave similar results to those observed with DPH (Fig. 34).

Figure 32: Fluorescence polarization vs temperature with DPH as probe for liposomes of: A) 14:0-PC (o------o) and 14:0-PSC (●------●), B) 16:0-PC (o------o) and 16:0-PSC (●------●), C) 18:0-PC (o------o) and 18:0-PSC (●------●), D) equimolar mixtures of 14:0-PC and 14:0-PSC (●------●) of 16:0-PC and 16:0-PSC (o------o) and of 18:0-PC and 18:0-PSC (■------■).

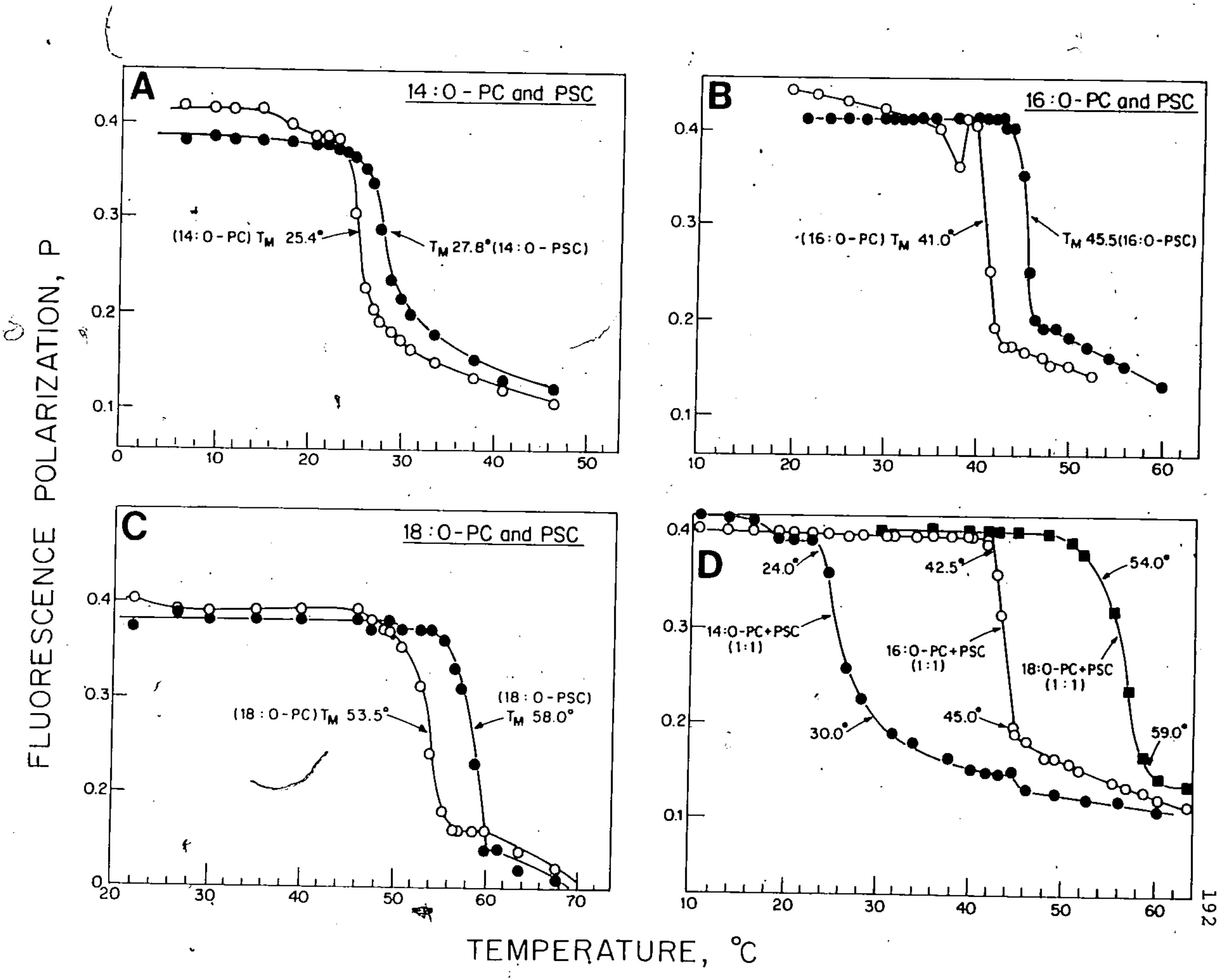

A
14 : 0 - PC and PSC
(14 : 0 - PC) T_M 25.4°
T_M 27.8° (14 : 0 - PSC)
B
16 : 0 - PC and PSC
(16 : 0 - PC) T_M 41.0°
T_M 45.5 (16 : 0 - PSC)
C
18 : 0 - PC and PSC
(18 : 0 - PC) T_M 53.5°
(18 : 0 - PSC) T_M 58.0°
D
24.0°
42.5°
54.0°
14:0-PC+PSC (1:1)
16:0-PC+PSC (1:1)
18:0-PC+PSC (1:1)
30.0°
45.0°
59.0°
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
FLUORESCENCE POLARIZATION, P
TEMPERATURE, °C

Figure 33: Fluorescence polarization as a function of temperature with cis and trans-parinaric acid probes, for liposomes of 16:0-PC (o-----o) and 16:0-PSC (●-----●)

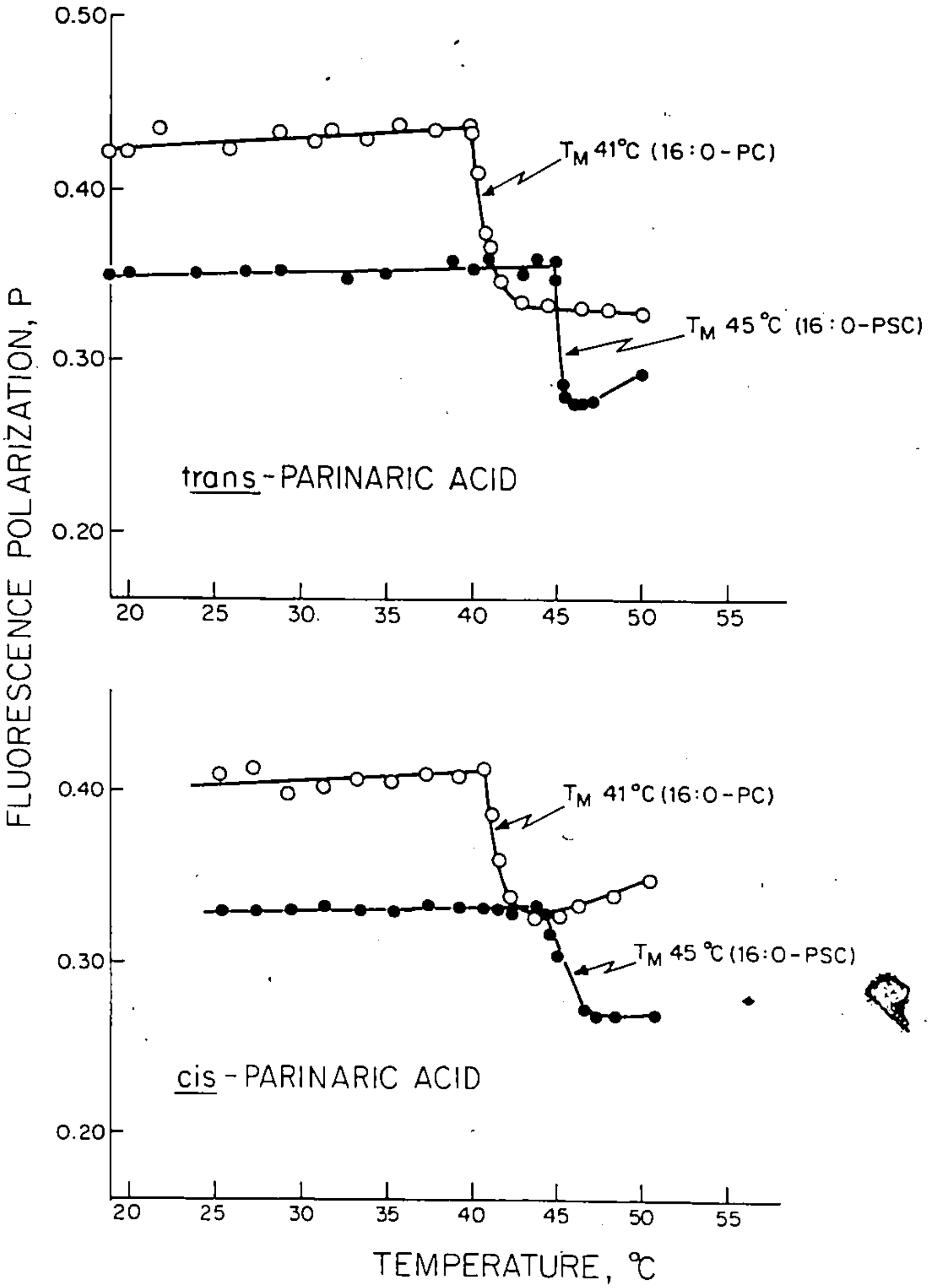
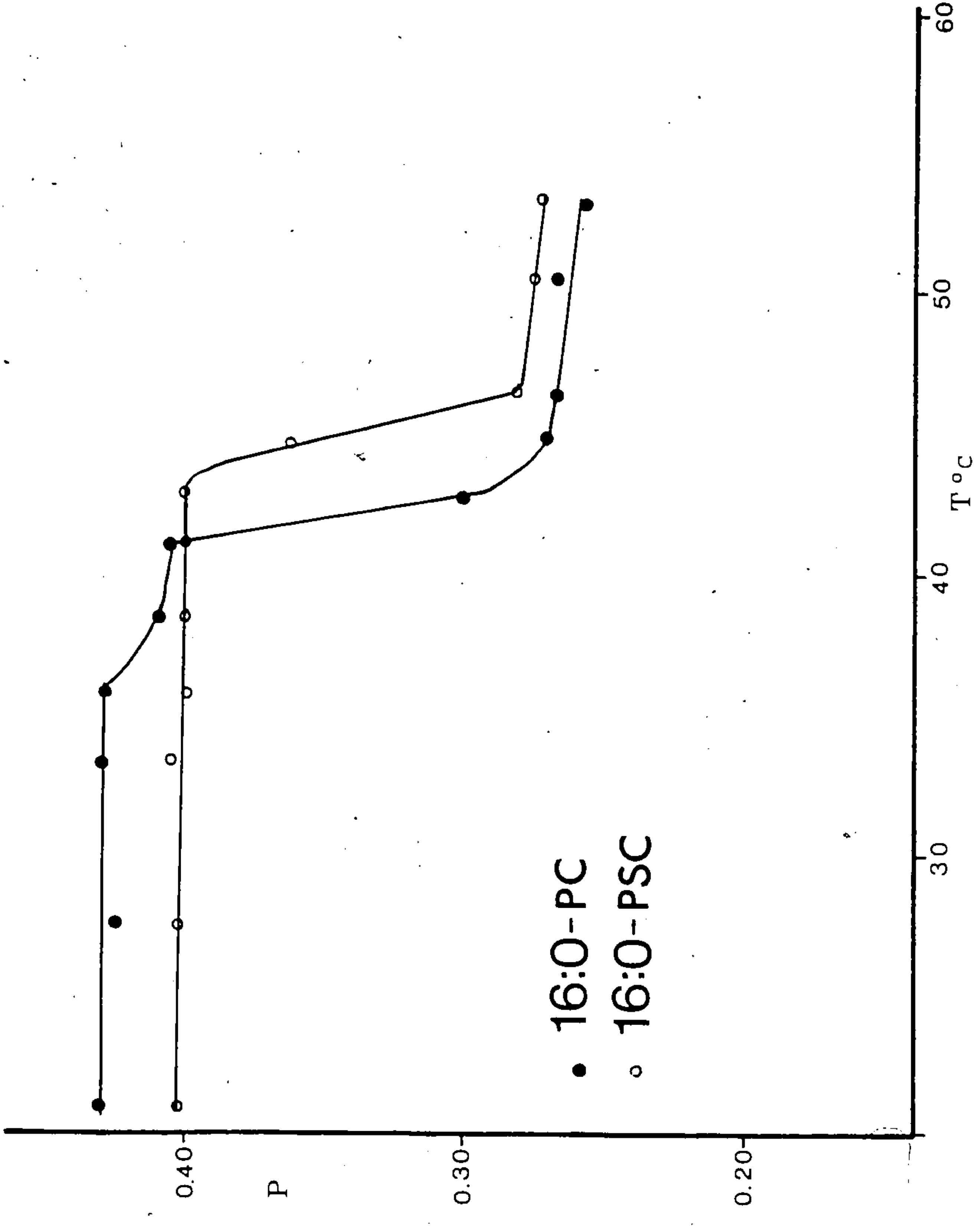

FLUORESCENCE POLARIZATION, P
0.50
0.40
0.30
0.20
T_M 41°C (16:0-PC)
T_M 45 °C (16:0-PSC)
trans-PARINARIC ACID
20
25
30
35
40
45
50
55
0.40
0.30
0.20
T_M 41 °C (16:0-PC)
T_M 45 °C (16:0-PSC)
cis-PARINARIC ACID
TEMPERATURE, °C

Figure 34: Fluorescence polarization as a function of temperature with para-quaterphenyl as probe for liposomes of 16:0-PC (●----●) and 16:0-PSC (o-----o).


● 16:0-PC
○ 16:0-PSC
P
0.40
0.30
0.20
T °C
30
40
50
60

2.5.4 Nuclear Magnetic Resonance

2.5.4.1 Deuterium

The deuterium NMR spectra of d-3-DPPSC, in the temperature range of 0-40 °C, appeared essentially as a single broad resonance whose magnetic field width diminished with increasing temperature (Fig. 35). This behaviour is similar to that found in other model membranes (e.g. d-9-DPPC) and biomembranes measured at temperatures below Tm, i.e. in the gel state (171, 163, 100). The rate of molecular motion and the degree of molecular order affects the spectral lineshape significantly. Unfortunately, it is difficult to separate the individual contributions of order and motion, even with detailed spectral lineshape simulation (172).

The half width of the signal increased from 3.62 KHz at 0 °C to 2.28 KHz at 40 °C and there was a concommitent change in the lineshape. The lineshape of the spectra between 40-50 °C showed a progessive change to a liquid-crystalline like appearance similar to that observed for other phosphatidyl cholines in the fluid state (173, 174, 175, 176). The quadrupole splitting, in the fluid state at 50 °C (5° above the Tm of d-3-DPPSC) was 1.03 KHz, a little lower than the value of 1.2 KHz reported for DPPC at 46 °C (176). A more accurate value of the quadrupole splitting of d-3-DPPSC at 50°C, calculated from the first moment, was 1.11 KHz, very similar to the value reported for DPPC.

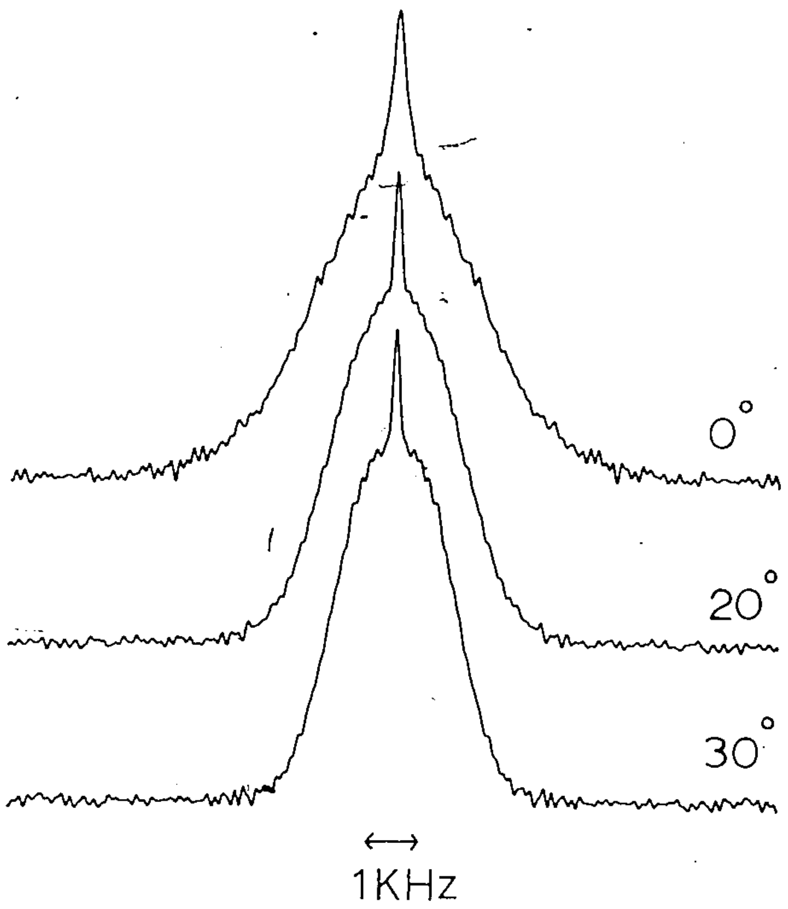
Figure 36 shows the variation in the first and second moments of the deuterium NMR spectra (related to the change in lineshape) of d-3-DPPSC. From 0-25 °C there was a progressive decrease in the moments, while between 30 and 40 °C there was a leveling off of the moments and finally between 40 and 50 °C there was a further decrease in the moments. The midpoint in the decrease in both the first and second moments between 42.5 and 47.5 °C was 45 °C. This value corresponds very well with the value obtained by calorimetry for this deuterated derivative.

The fraction of the lipid in the gel state or the liquid-crystalline state at the temperatures just below and above the transition temperature, calculated from the first, second and third moments, by the method of Jarrell et al (164), was as follows: DPPSC up to 40 °C was all in the gel state, while it was completely fluidized by 48 °c. The midpoint in the melt (44 °c), where 50 % of the lipids are in the gel state and 50 % are in the liquid crystalline state is again in good agreement with the values obtained by the other methods.

2.5.4.2 Phosphorus NMR

The ^{31}P NMR spectra of DPPSC (Fig. 37) had the expected lineshape for a bilayer structure with rapid averaging (on the scale of ^{31}P chemical shift anisotropy) of the axial chemical shift tensor elements (177, 173). An increase in the rate of motion of the phosphate containing head groups, with increasing temperature is shown by the improved definition of the lineshapes at the higher temperature. The values of the chemical shift anisotropy (CSA) in the gel state and liquid-crystalline states were 55 and 44.6 ppm respectively slightly lower but comparable to similar data for DPPC at 58 and 49 ppm (176). The slightly lower values for DPPSC are indicative of a larger rate of motion and/or amplitude for the phosphate head group moiety. Figure 38 shows the variation of the residual chemical shift anisotropy with temperature. The transition temperature at 45 °C is in good agreement with the value obtained by all the other methods (Table 5).

Figure 35: ^{2}H NMR spectra (46.1 MHz) of dipalmitoyl phosphatidyl-S- methyl-^{2}H -S-methyl-^{1}H -mercaptoethanol dispersed in excess deuterium-depleted water at the indicated temperatures. The plots are 15 kHz in width.


0°
20°
30°
1KHz

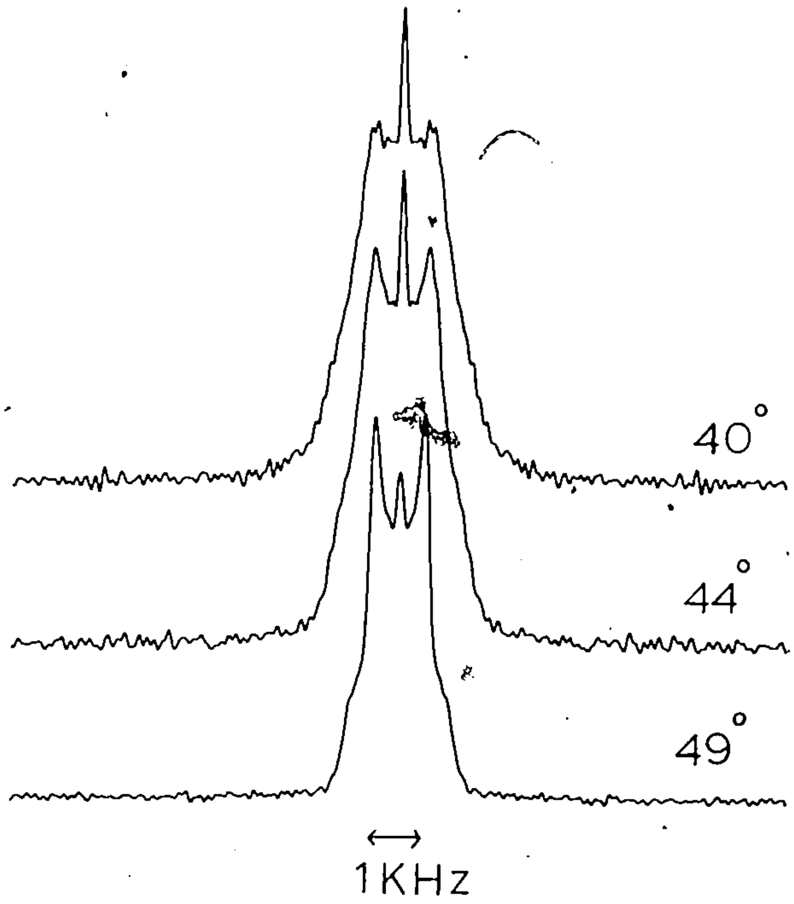

40°
44°
49°
1KHz

Figure 36: Variation with temperature of the first and the second moments of the ^2H NMR spectra of 16:0-PSC, and the fraction of the liquid crystalline lipid (164).

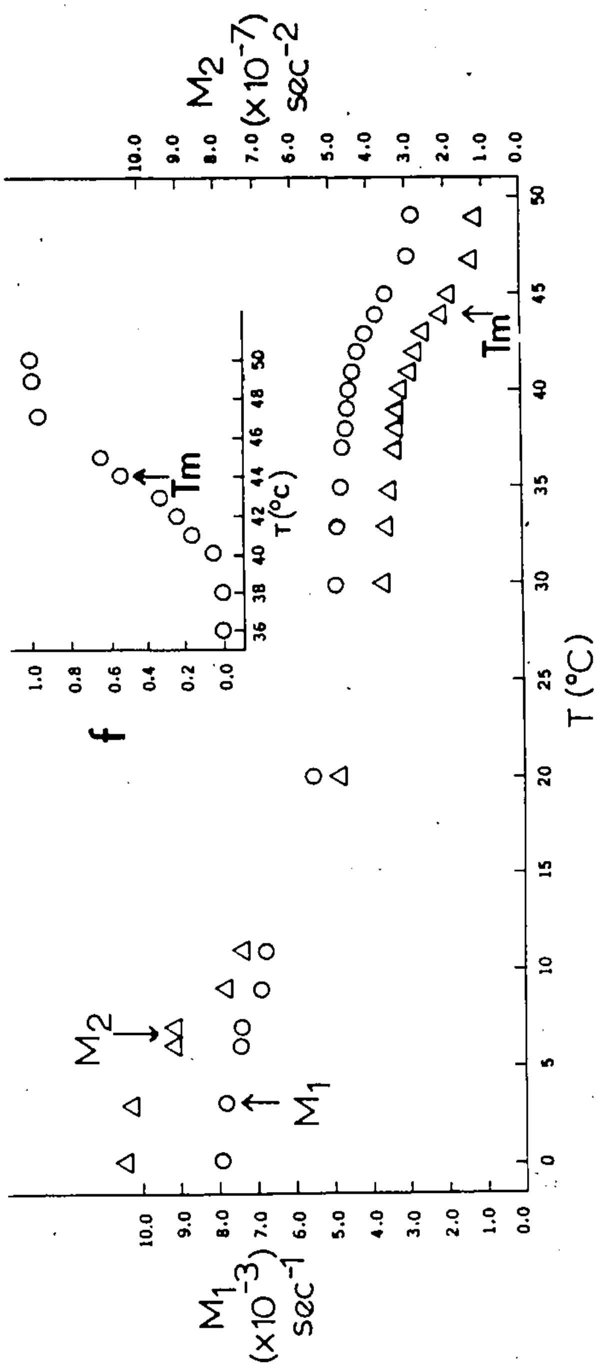
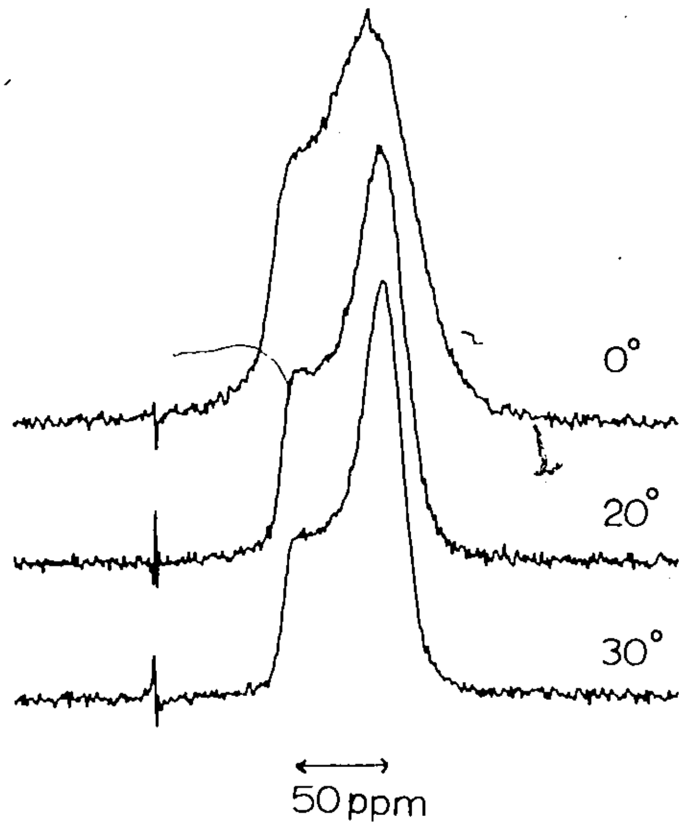

M_1 $(\times 10^{-3})$ sec^{-1}
M_2 $(\times 10^{-7})$ sec^{-2}
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
T (°C)
0
5
10
15
20
25
30
35
40
45
50
M_1
M_2
Tm
f
0.0
0.2
0.4
0.6
0.8
1.0
T (°C)
36
38
40
42
44
46
48
50
Tm

Figure 37: ^{31}P NMR spectra (121.46 MHz) of 16:0-PSC dispersed in excess water at the indicated temperatures. The plots are 45 KHz wide.


0°
20°
30°
50ppm

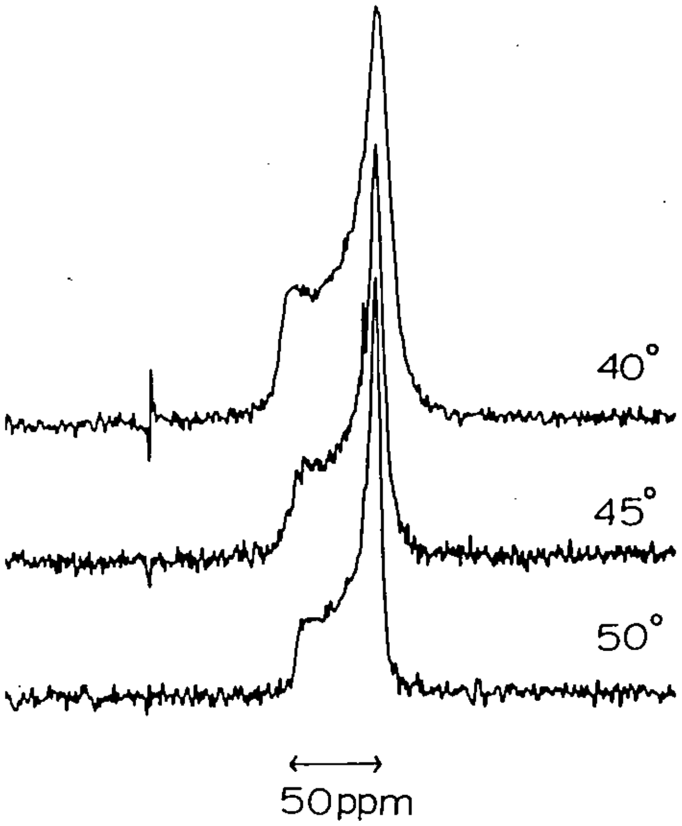
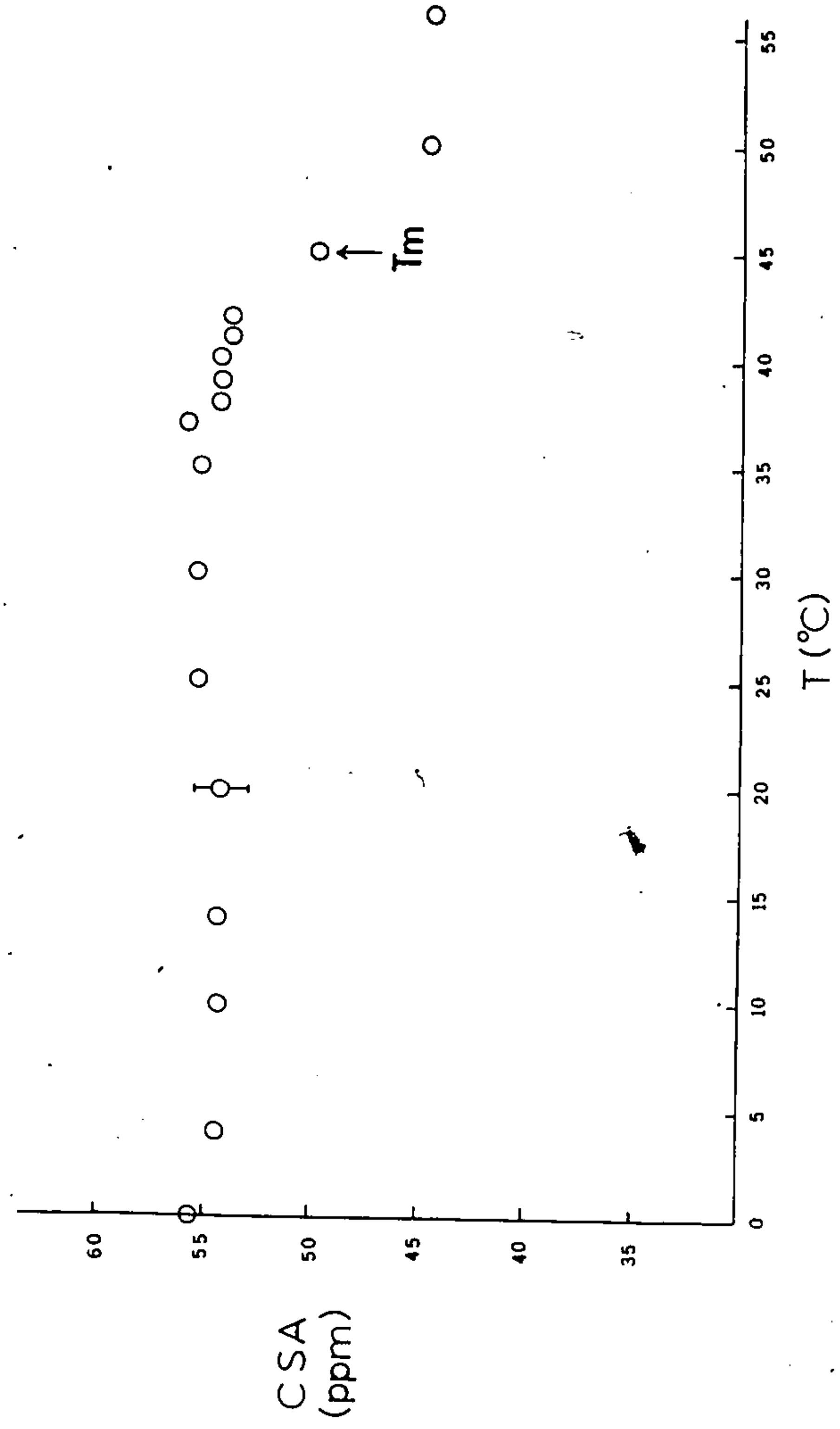

40°
45°
50°
50ppm

Figure 38: Variation with temperature of the residual CSA of the ^{31}P NMR spectra of 16:0-PSC measured from the inflection point on the low field shoulder to the maximum at the high field.


CSA (ppm)
60
55
50
45
40
35
T (°C)
0
5
10
15
20
25
30
35
40
45
50
55
Tm

2.5.5 Fourier Transform Infrared Spectroscopy

In the spectra of dispersions of DMPSC the large excess of water was evident from the strong IR absorption in the O-D stretching region (2700-2300 cm^{-1}). The major infrared absorption bands were identical for all three derivatives, except for the increase in the intensity of the C-H stretching bands relative to the C=O stretching band in the PSCs with longer fatty acids. The spectra of the PSCs were similar to the spectra of the PCs except that the sulfocholine methyl bands were at 3023 ($C H_2$ stretch) and 1435 cm^{-1} ($C H_2$ deformation) whereas the corresponding choline methyl bands were at 3040 and 1490 cm^{-1} (103).

Three IR absorption bands were of high diagnostic value: the "factor group" splitting of the methylene scissoring mode at 1470 cm^{-1}, the CH_2 stretching vibrations at 3100-2800 cm^{-1} and the ester C=O stretching vibration at 1735 cm^{-1}.

For example, in the spectra of the C-H stretching region of DMPSC changes in frequency, peak height and band shape were evident as a function of temperature and phase changes. The symmetric $(CH_2)n$ stretching band for DMPSC, DPPSC and DSPSC showed a frequency and bandwidth dependence on temperature, and phase change (Fig. 39 A, B) with thermotropic behaviour similar to that of other phosphatidyl cholines, which showed large and abrupt changes in the frequency and bandwidth at the gel to liquid-crystalline transition. The sharp increase in frequency and bandwidth at the Tm reflected the increased conformational disorder of the acyl chains in the phase transition. Identical plots were obtained for the antisymmetric $(CH_2)n$ stretching bands at 2920 cm^{-1} (Fig. 41). As with other phospholipids, the frequency and bandwidth of the CH_2 stretching modes were usefull for characterisation of the Tm, but were rather insensitive to solid:solid phase transitions such as the pretransition (Tp).

The $(CH_2)n$ scissoring mode on the other hand has been used to monitor the nature of the packing of the fatty acyl chains in the gel phase. This methylene bending mode at 1470 cm^{-1} results from the out of phase scissoring vibration of the methylene groups in the all trans segments of the acyl chains. The $(CH_2)n$ scissoring modes of DMPSC at low temperature (e.g. 1470 cm^{-1}, 5 °C) showed group splitting or crystal field splitting which indicated the presence of a rigid orthorhombic subcell (102). The splitting decreased with increasing temperature until Tp where only one peak remained (1468 cm^{-1}) (Fig. 40). In DPPSC and DSPSC the CH_2 scissoring mode was split in two bands at all temperatures in the gel phase below the Tm but the peaks seemed to coaless into a simgle peak just below the Tm, indicating the possibility of a pretransition in these derivatives.

The bandwidth of the CH_2 scissoring band (1470 cm^{-1}) has also been used to demonstrate the pretransition behaviour (102). Thus the bandwidth for DMPSC decreased with increasing temperature up to Tp, and thereafter the bandwidth remained relatively constant up to the Tm. At the Tm there was a step increase in the bandwidth, typical of the gel to liquid-crystalline phase change. Similar changes in bandwidth were apparent in DPPSC and DSPSC spectra. However the occurence of a Tp cannot be clearly defined.

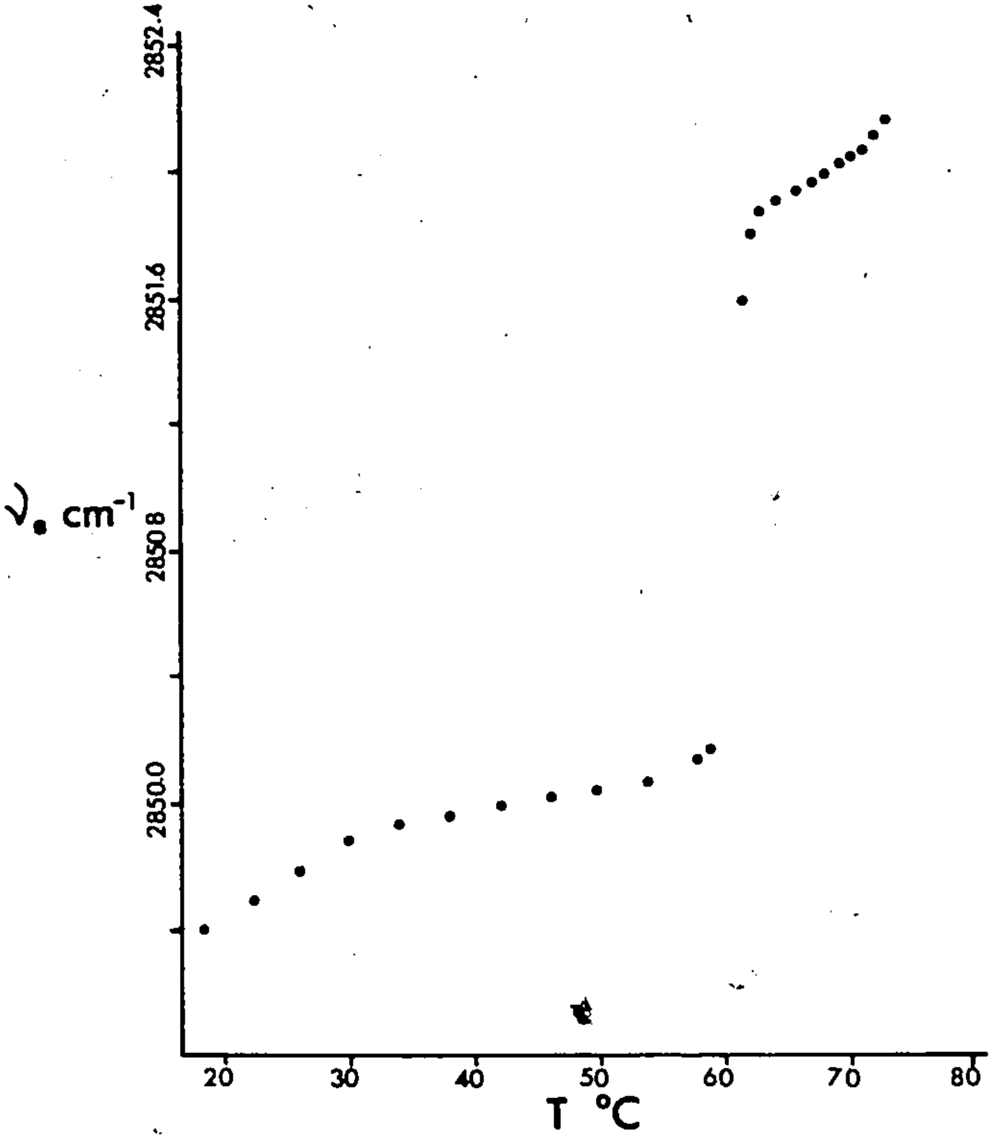
Further evidence for a Tp in DMPSC was obtained from the changes in frequency of the sn1 and sn2 ester bands, (Fig. 42).

The absorption bands of the hydrophilic groups were rather insensitive to temperature and phase changes, probably due to the large degree of hydration of these groups (80).

All three derivatives also showed evidence of a subtransition at a temperature far below the Tm. The nature of this transition is being investigated further as its behaviour seems quite different from that of PC.

Figure 39: The temperature dependence of the frequency (A) and the bandwidth (B) of the methylene stretching band of 18:0-PSC.

A



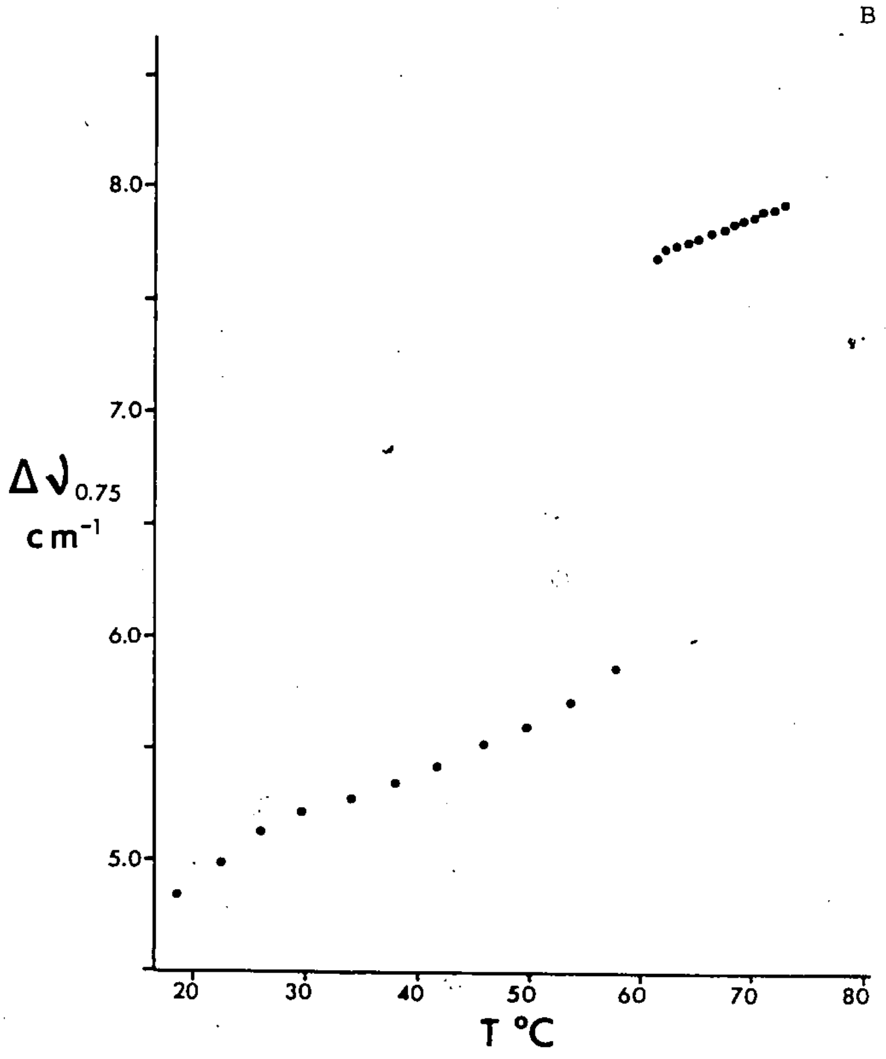

B
8.0
7.0
6.0
5.0
Δν 0.75
cm⁻¹
20
30
40
50
60
70
80
T °C

Figure 41: Temperature dependence of the CH_2 stretching bands (symmetric and antisymmetric) of 14:0-PSC in 2 °C steps between 10 and 30 °C.

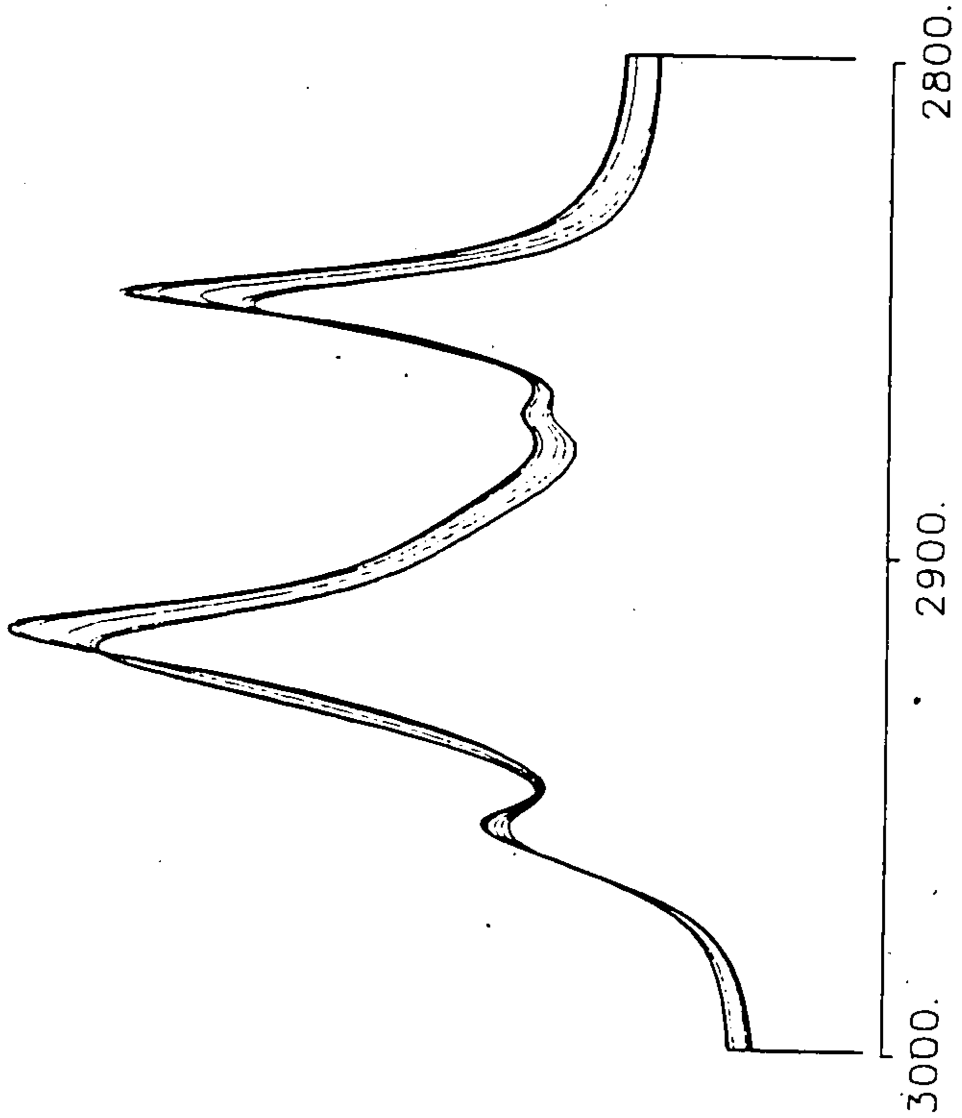

3000.
2900.
2800.
WAVENUMBER, CM^{-1}

Figure 40: Difference spectra of 16:0-PSC between 1500-1440 cm^{-1} as a function of temperature.

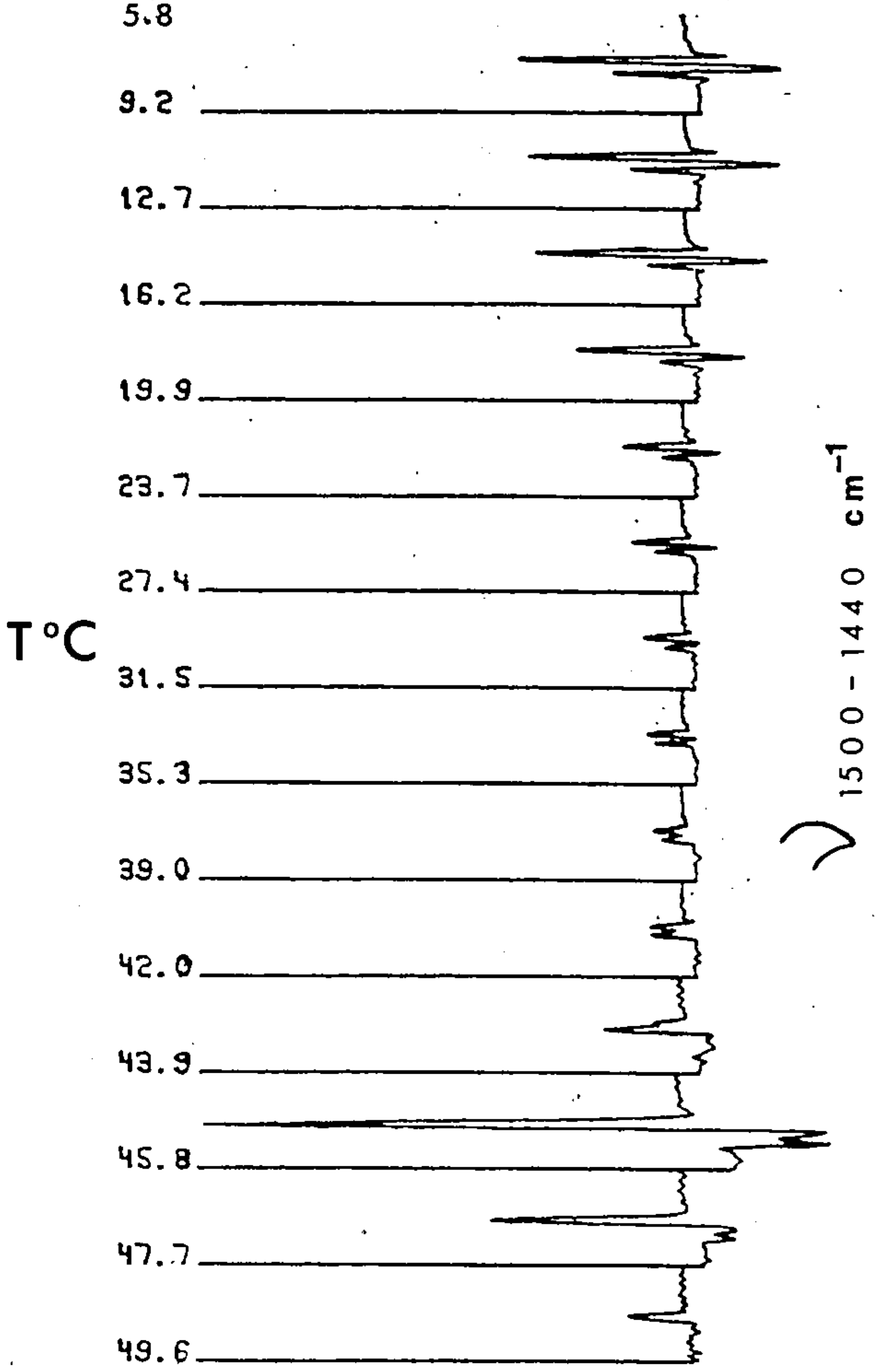
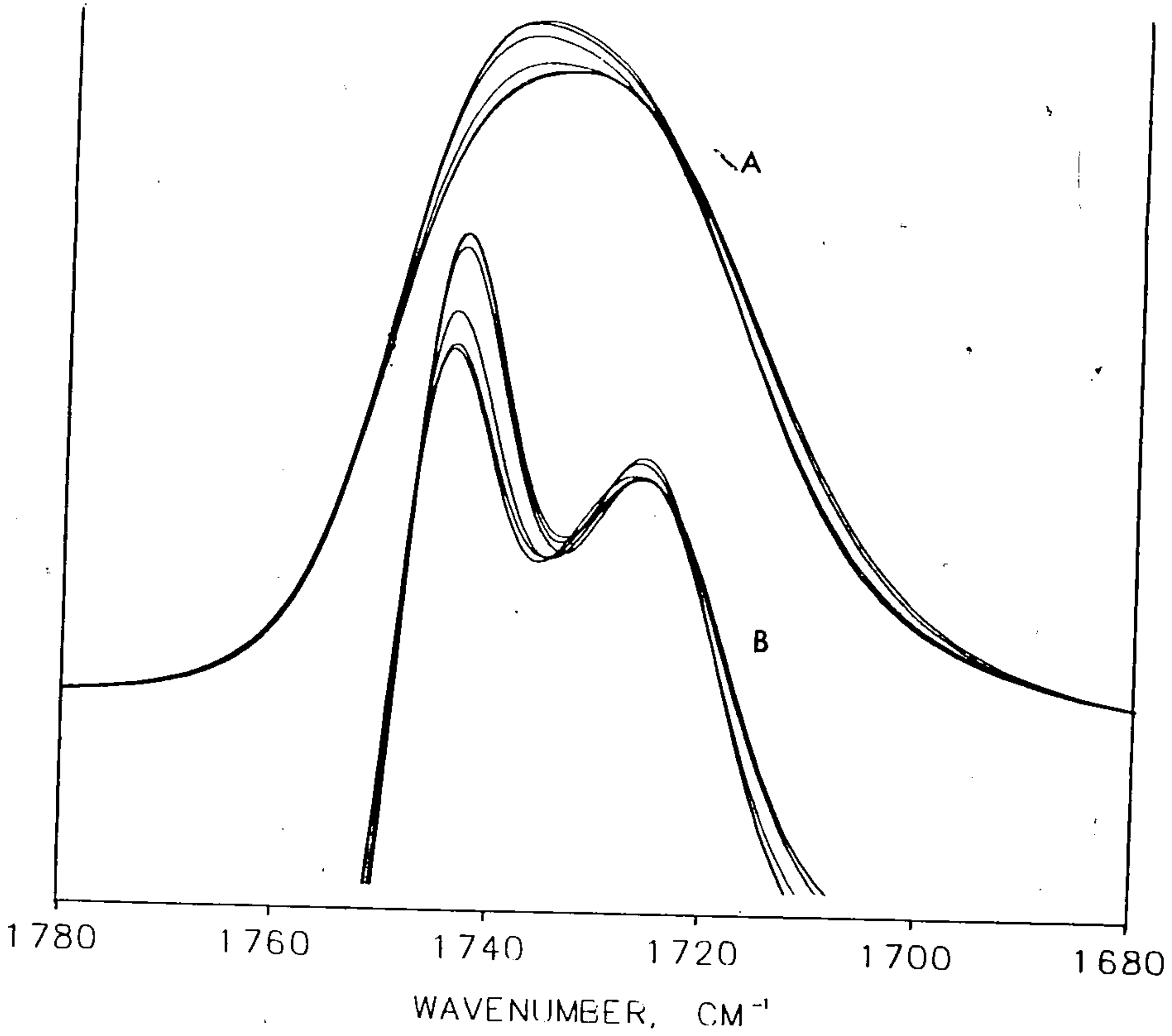

5.8
9.2
12.7
16.2
19.9
23.7
27.4
T°C
31.5
35.3
39.0
42.0
43.9
45.8
47.7
49.6
ν 1500 - 1440 cm⁻¹

Figure 42: Temperature dependence of 14:0-PSC carbonyl stretching band A) as measured, B)after Fourier self-deconvolution (183) in 2° steps between 18- 28°C.

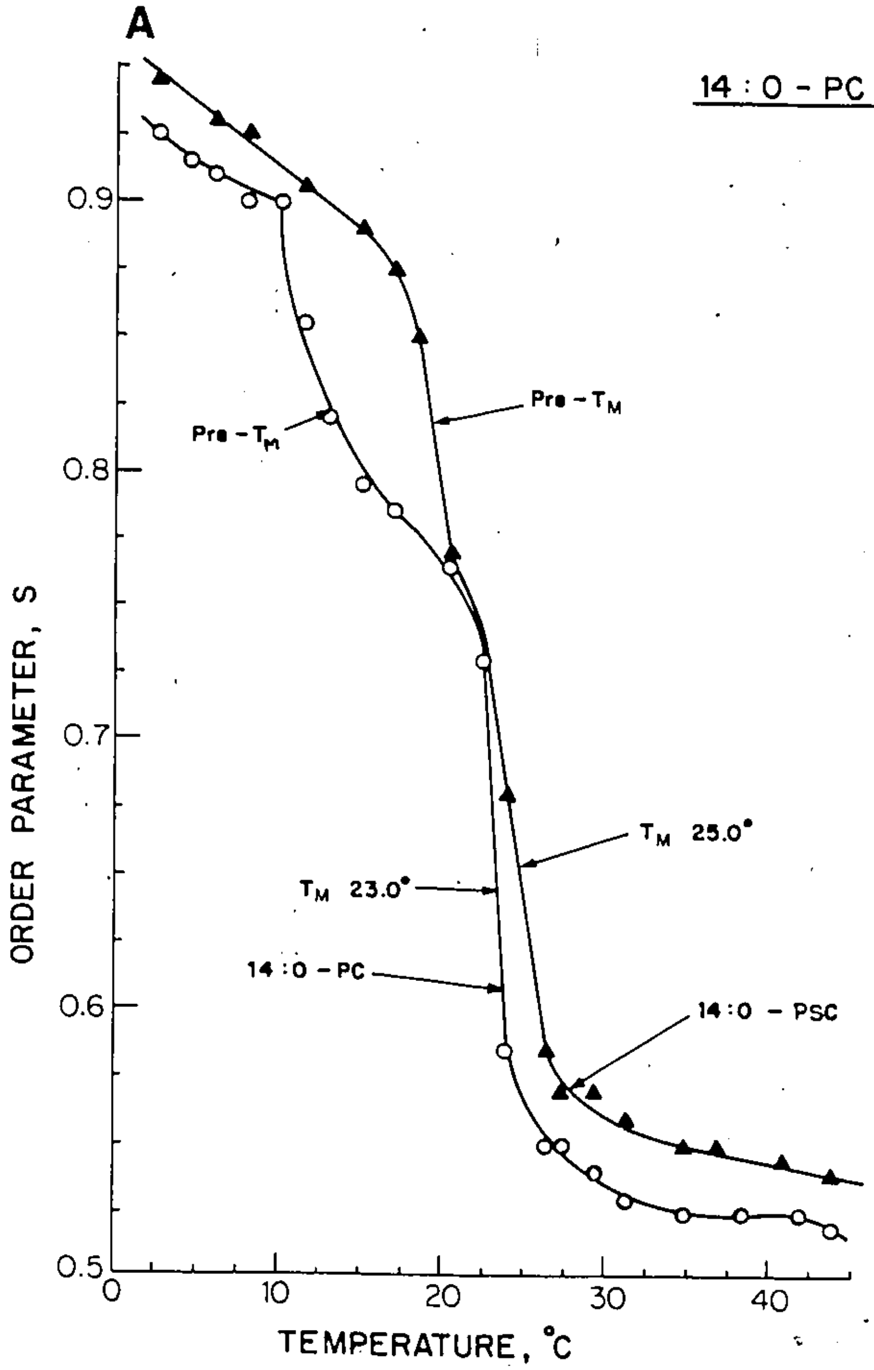

A
B
1780
1760
1740
1720
1700
1680
WAVENUMBER, CM⁻¹

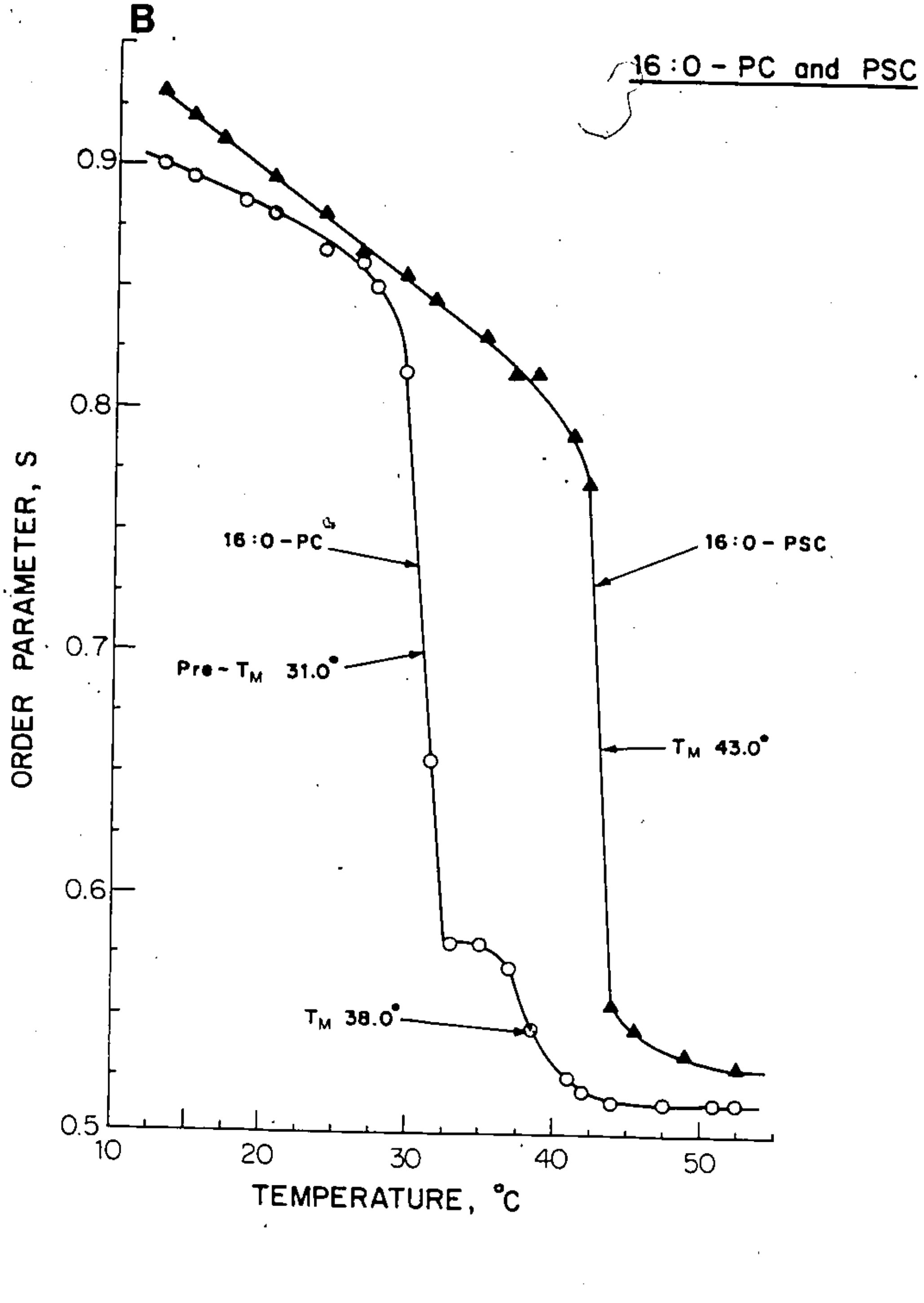
2.5.6 Electron Paramagnetic Resonance Studies

With the 5-doxyl stearic acid as probe, somewhat lower Tm's were measured for all PCs and PSCs (Table 5 & Fig. 43 most likely due to perturbation of the bilayer structure by the bulky doxyl probe. In addition, the 5-doxyl stearic acid probe gave greater differences in transition temperature between PSCs and the corresponding PCs than was found by DSC or by fluorescence polarization. At low temperatures, in the gel state, the order parameter was close to the value of 1.0 for all PCs and PSCs, indicating a high degree of order and a highly rigid system. However in contrast to the fluorescence studies the value of S of the PSCs was consistently somewhat higher in both the gel and fluid states than the S values of corresponding PCs and a steady decline in S was observed with increasing temperature. All the PC samples, particularly DPPC, showed a pretransition which resulted in a surprisingly large drop in the order parameter (Fig. 43 B). In contrast to the results with the PC samples, only di-14:0 PSC showed any pretransition (Fig. 43 A) and the magnitude of the drop in S was much less than for the PCs. Curiously, di-18:0 PC in the liquid-crystalline state showed a much greater degree of disorder (S= 0.25) than the corresponding PSC (Fig. 43 C). The difference in the transition temperature between di-18:0 PC and di-18:0 PSC was accordingly very large (10 °C), in contrast to the much smaller differences measured by DSC or fluorescence.

The Tm measured with the probe N-tempo stearamide with aqueous dispersions of DPPSC gave a Tm at 45 °C similar to that found in calorimetry (Fig. 44).

Figure 43: EPR spectra as function of temperature, with 5-doxylstearic acid for liposomes of: A) 14:0-PC (o---o) and 14:0-PSC (▲---▲), B) 16:0-PC (o---o) and 16:0-PSC(▲---▲) C) 18:0-PC (o---o) and 18:0-PSC (▲---▲).


A
14 : 0 - PC and PSC
ORDER PARAMETER, S
TEMPERATURE, °C
0.5
0.6
0.7
0.8
0.9
0
10
20
30
40
Pre - T_M
Pre - T_M
T_M 25.0°
T_M 23.0°
14 : 0 - PC
14 : 0 - PSC


B
16 : 0 – PC and PSC
16 : 0 – PC
16 : 0 – PSC
Pre – T_M 31.0°
T_M 43.0°
T_M 38.0°
ORDER PARAMETER, S
0.9
0.8
0.7
0.6
0.5
10
20
30
40
50
TEMPERATURE, °C

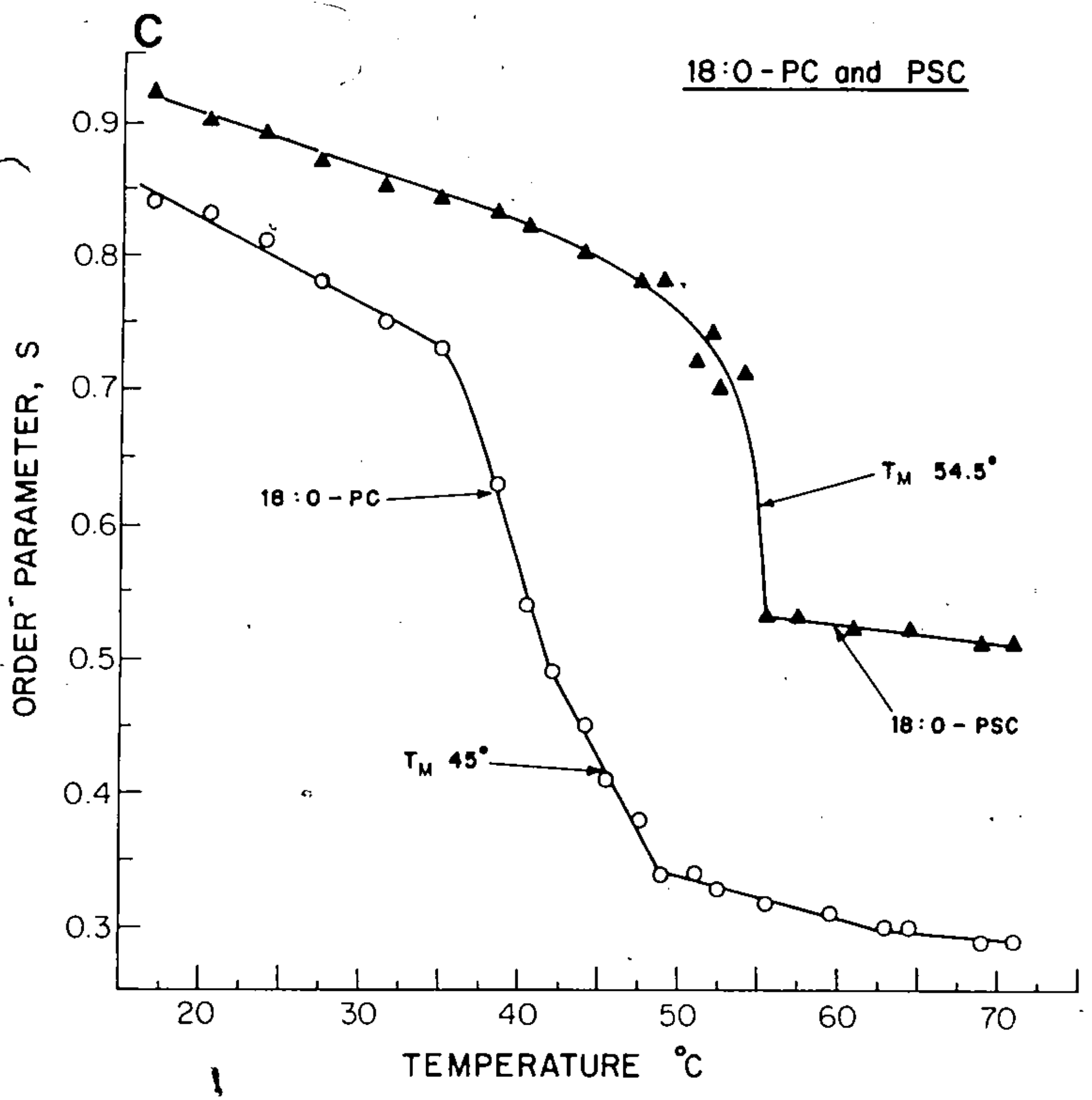
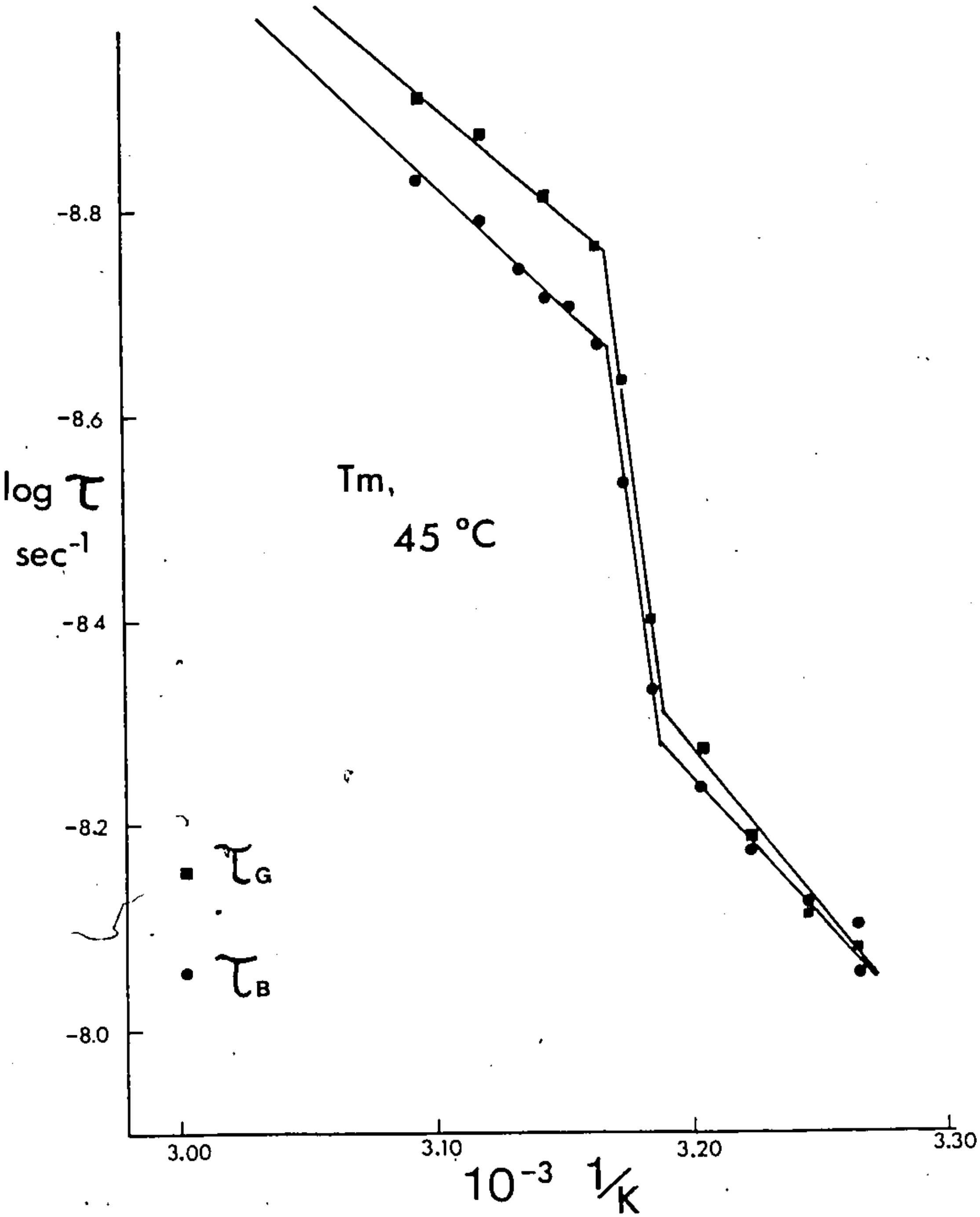

C
18:0-PC and PSC
ORDER PARAMETER, S
0.9
0.8
0.7
0.6
0.5
0.4
0.3
20
30
40
50
60
70
TEMPERATURE °C
18:0-PC
T_M 45°
T_M 54.5°
18:0-PSC

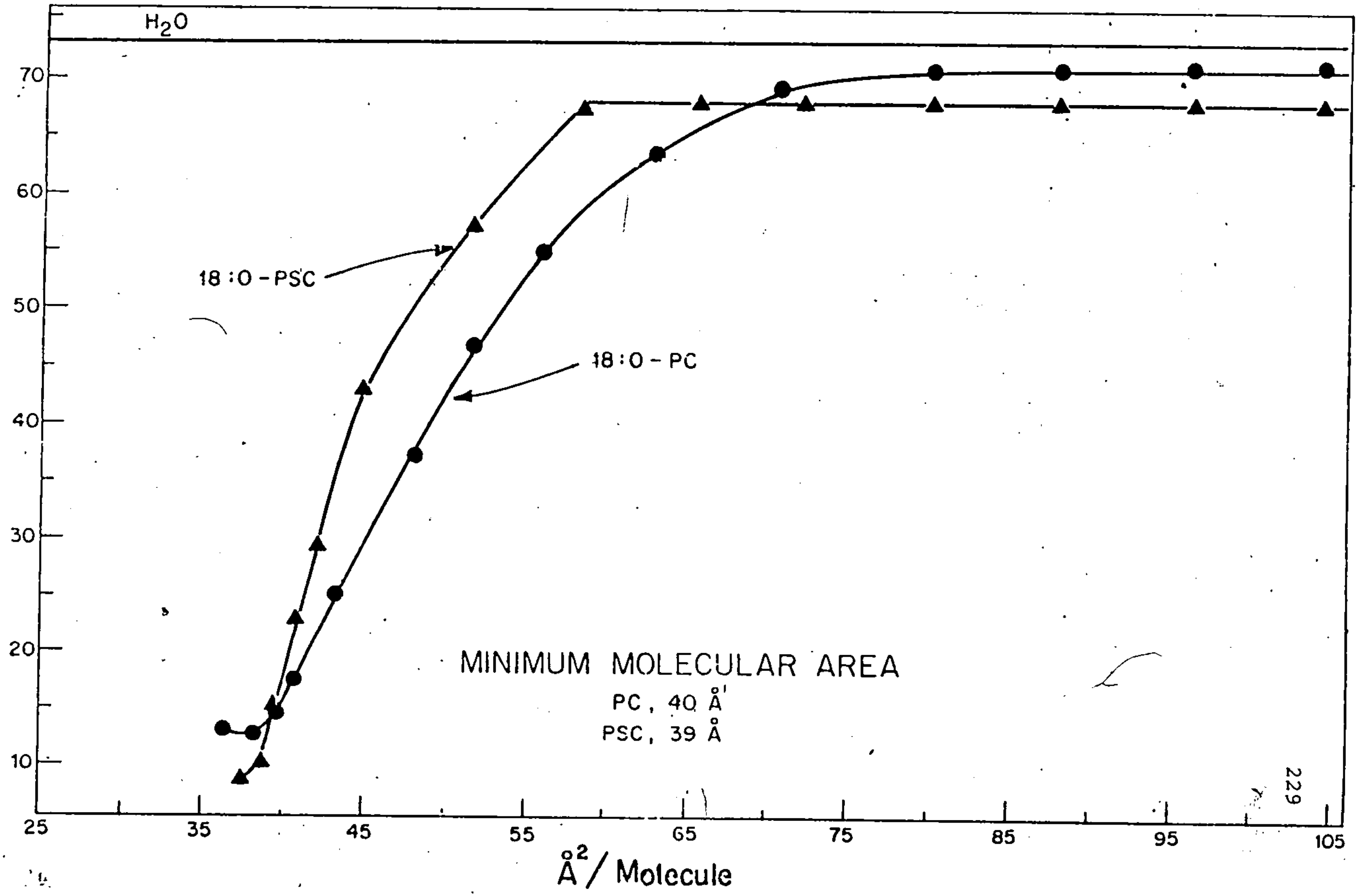
Figure 44: Electron spin resonance as a function of temperature, with N-tempostearamide probe for liposomes of: A) 16:0-PC (●-----●) and 16:0-PSC (■-----■).


log τ
sec^{-1}
-8.8
-8.6
-8.4
-8.2
-8.0
Tm,
45 °C
■ τ_G
● τ_B
3.00
3.10
3.20
3.30
10^{-3} 1/K

2.5.7 Monolayer Studies

The expanded region of the film, where the surface tension is independent of surface area, occurred above 78 A ²/molecule and 58 A ² /molecule for PC and PSC, respectively. In this region, the surface tension of the PSC monolayer was slightly lower than that of the PC monolayer indicating that the head groups of PSC in the phase transition interact more strongly with water than with the head groups of PC. The fact that the onset of the compressible liquid region in the PSC monolayer occurred at a more condensed state compared to the PC monolayer indicated that PSC does not aggregate as readily as PC. However, the higher surface tension of PSC, compared to PC at any point in the liquid state indicates that PSC head groups interact less strongly with water and more strongly with each other than the PC head groups. However, the minimum cross-sectional areas of PSC and PC, at the collapse point, were identical within experimental error (39 A² and 40 A², respectively).

Figure 45: Surface tension as a function of monolayer film area of 18:0-PSC (▲------▲) and 18:0-PC (●------●).


H_2O
70
60
50
40
30
20
10
25
35
45
55
65
75
85
95
105
18:0-PSC
18:0-PC
MINIMUM MOLECULAR AREA
PC, 40 Å
PSC, 39 Å
$\mathring{A}^2$/ Molecule

2.6 DISCUSSION

2.6.1 Calorimetry

The similarity in the thermal behaviour of PSCs and corresponding PCs may be explained by the similar size and shape of their analogous structures. The sharp gel to liquid crystalline phase transition of PSCs was an indication of the high degree of cooperativity in the melting of the fatty acyl chains as in the PCs. However the higher transition temperatures (Tm) observed for the saturated PSCs compared to those of the corresponding PCs may be explained by considering the relative size, state of hydration and intermolecular interactions of their respective head groups. As is evident from examination of space filling molecular models (Fig. 46) and from van der Waals radii calculations (182), replacement of the $-N^{+}(CH_3)_3$ group in PC by $-S^{+}(CH_3)_2$ to form PSC, results in an increased swept out volume of the polar head (Table 7). This increase may be estimated to be 20 % because the higher van der Waals radius of the sulfur compared to nitrogen more than compensates for the additional methyl group in choline. Since PC emulsifies a greater quantity of water than PSC does, it may be said that the polar head of PC is hydrated to a greater degree than that of PSC; thus less interaction would be expected between neighboring phosphocholine groups in PC than between phosphosulfocholines groups of PSC. This difference in behaviour parallels that of PC and PE as discussed by Boggs (87) (see subsection 2.2.5). Thus, in the gel phase, intermolecular forces between the PSC head groups are greater than between the PC head groups resulting in a more stable gel phase requiring a slightly higher temperature to melt the acyl chains. The identical change in enthalpy or entropy of melting for PCs and PSCs is expected considering that they are a measure of the same process namely melting of the acyl chains.

The difference in the pretransition temperature of the saturated PSCs compared to the corresponding PCs is consistent with the above theory. A smaller temperature difference between Tp and Tm is expected for a more "interactive" head group like PSC which should lead to a greater stability in the packing of the acyl chains and to a higher Tp (Fig. 47).

The lower transition temperature of di-18:1 PSC as compared to di-18:1 PC seems to contradict the above argument. However, the poorer packing of the unsaturated oleyl chains compared to saturated chains, may lead to less favorable packing of the head group of PSC than of PC and to corresponding lower Tm for di-18:1 PSC. In other words, because of the geometry difference between the two polar heads, the positive or negative charges of the larger sulfonium head groups may come into proximity with similar charges on the neighboring head groups and lead to a greater general destabilization of the bilayer structure and to a lower transition temperature for the unsaturated di-18:1 PSC than for di-18:1 PC.

Cholesterol was observed to interact with di-16:0 PSC in the same way as with the analogous PC, the gel to liquid crystalline transition being abolished at a mole ratio of 1:1 (Fig. 31) (2). Such interaction appears not to involve any strict structural requirement in the polar head group (112). Thus any 'onium' polar head group (e.g. sulfonium, ammonium, arsomium, phosphonium, etc.) should be able to substitute for the quaternary ammonium group in PC. This conclusion is in accord with the results presented elsewhere that show that di-14:0 PSC and di-14:0 PC have similar permeability properties in the presence of cholesterol (112).

It appears then that the hydrocarbon chain interactions are not greatly influenced by PSCs somewhat larger polar head group than PCs'. These results suggests that the PSC head group may be less tilted with respect to the plane of the hydrocarbon chains than the PC head group.

Figure 46: Space filling molecular models of PC (top) and PSC (bottom).

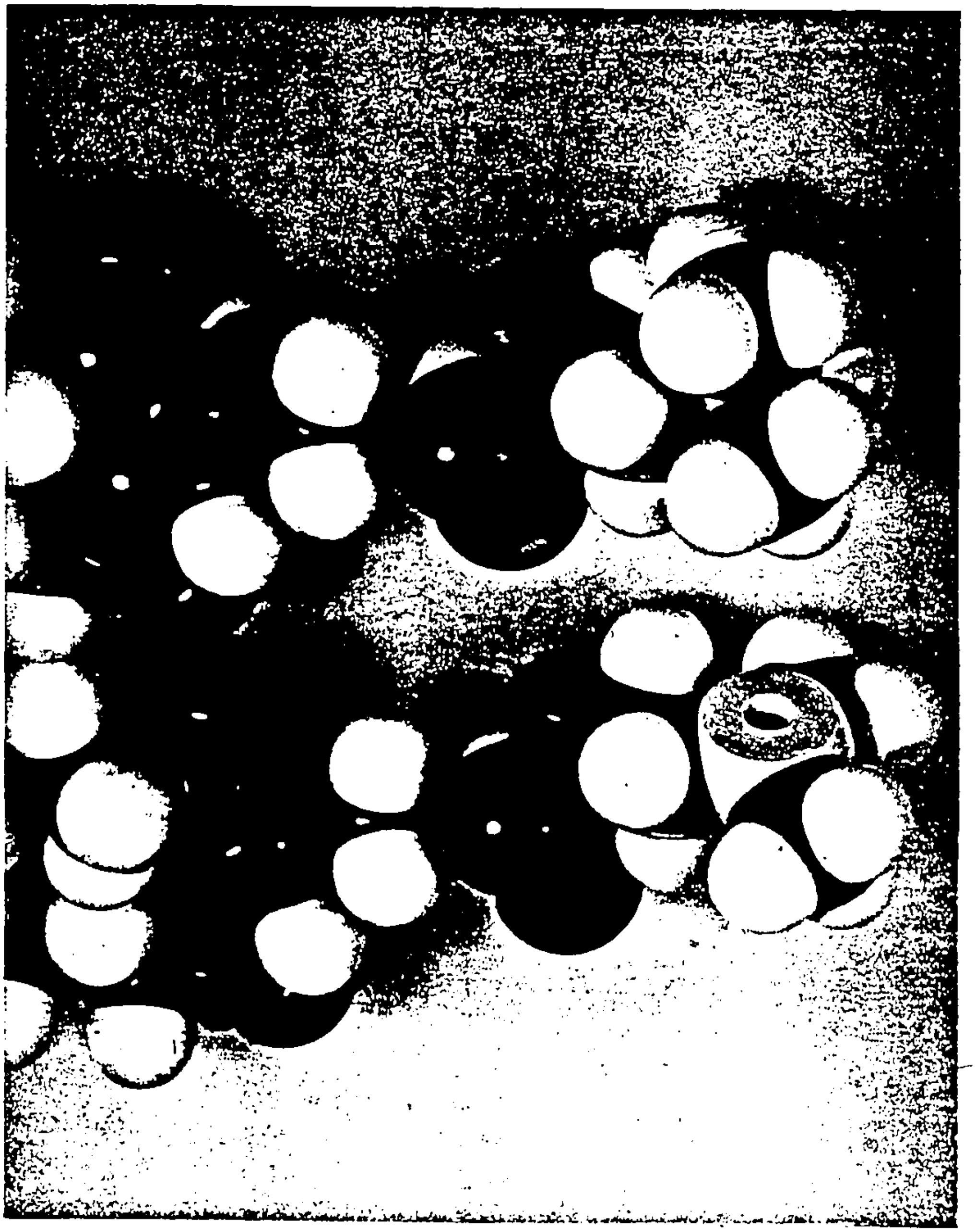
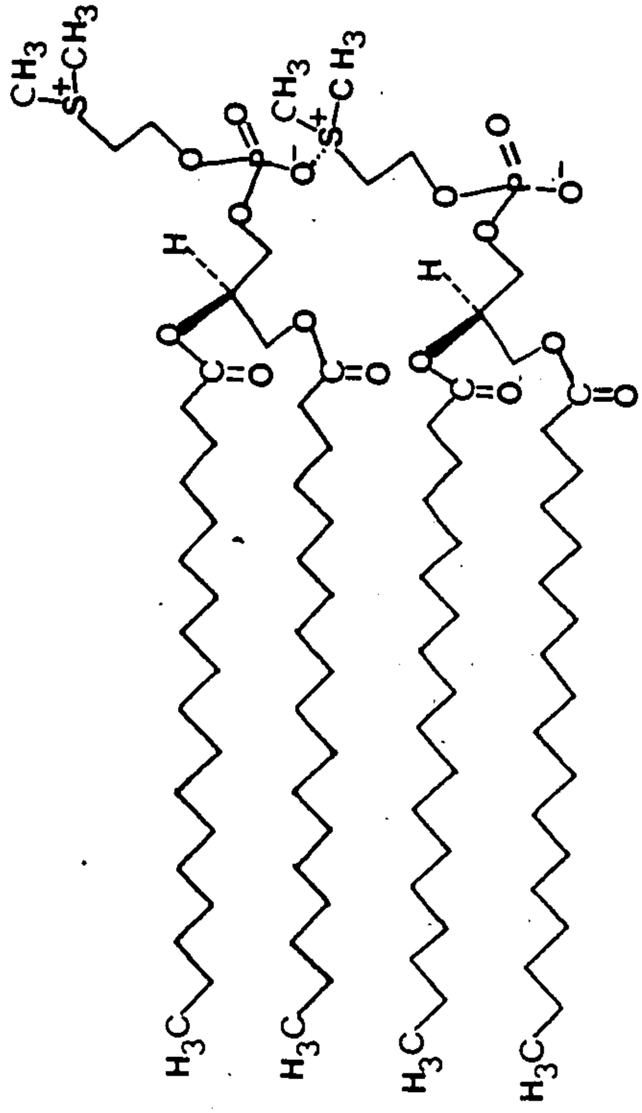


Figure 47: Postulated packing of two 18:0-PSC molecules in a bilayer


CH3
S+
CH3
O
P
O−
CH3
S+
CH3
O
P
O−
H
H
C=O
C=O
C=O
C=O
H3C
H3C
H3C
H3C

7

TABLE 7

Calculation of the ratio of the swept-out volumes of the of the sulfonium group and the ammonium groups of PSC and of PC, respectively, using van der Waals radii (182)

DIMENSIONS OF SULFOCHOLINE AND CHOLINE GROUPS[a]

Analogue	Width ^{o}A	Length ^{o}A	Depth ^{o}A	Volume swept out[b] ^{o}A	Volume ratio ^{o}A
Ammonium	6.2	5.5	6.0	166	1.00
Sulfonium	6.6	6.0	4.5	205	1.24

[a] Average value of dimension determined by measurement of spacefilling models.

[b] Volume of cylinder swept out = $\frac{\pi\,(\text{width})^2}{2}$ X length

2.6.2 Steady State Fluorescence Polarization

The transition temperatures (Tm) obtained from the steady state fluorescence polarization using DPH were in agreement with the calorimetric results (Table 5).

The slightly higher fluorescence polarization intensities in the gel phase of the PCs relative to the PSCs observed with DPH as probe (Fig. 32) would indicate a slightly less ordered gel state in the central hydrocarbon core region of the PSC bilayer. With the cis and trans parinaric acid probes, which report from the hydrocarbon region close to the polar head group (152), the differences between the polarization intensities of PC and PSC were more pronounced, both in the gel and the liquid crystalline states (Fig. 33). These results suggest that the larger sulfocholine group introduces slightly more space between the hydrocarbon chains in the region of the polar head, thus increasing the distances between the methylene groups in this area. A smaller effect would exist further down in the central hydrocarbon core and would account for the fact that the differences in polarization intensities between PCs and PSCs are not as high with the DPH probe as with the parinaric acid probes (Figs 32 and 33).

2.6.3 Electron Paramagnetic Resonance

The lower transition temperatures observed by EPR with the 5-doxyl stearic acid probe compared to DSC and fluorimetry (Table 5) are more likely due to the perturbing effect of the large nitroxide group in the spin probe. Nevertheless, the PSC analogues consistently showed 2-4°C higher transition temperatures than the corresponding PCs, as was found with DSC and fluorimetry. However, it should be noted that the order parameters for PSCs in both the gel and liquid crystalline states are higher than for the corresponding PCs (Fig. 43), which is the reverse of that observed with the fluorescent probes (Figs 32 and 33). A possible explanation might be that the larger spaces between the hydrocarbon chains in the region of the polar head in the PSCs, discussed above, would allow the large 5-doxyl spin probe to intercalate with the chains in a less perturbing manner than in the corresponding PCs. The greater disruption of PC structure with the longer fatty acyl chains indicates that the packing of the longer chains is correspondingly tighter.

2.6.4 Fourier Transform Infrared

The gel to liquid-crystalline transition of the saturated PSCs was confirmed by large concomitant variations in peak height, frequency and bandwidth similar to changes observed in the melting of DPPC and n-alkanes (178). Similar but smaller changes in the spectra of the PSCs in the liquid-crystalline state were an indication of a progressive temperature dependent increase in the average number of gauche conformers in the acyl chains with higher temperature.

The nature of the gel state could be studied by examining several bands arising from the acyl side chains. The most diagnostic band concerning the subcell unit of the acyl chain packing was the methylene scissoring band. Thus, the large group factor splitting of this band at very low temperatures (-80 °C) was indicative of a tight orthorhombic or monoclinic subcell (Fig. 24). Since the acyl chains of saturated lecithins pack in orthorhombic arrangement at these low temperatures in the gel state, it is probable that the same crystal lattice structure occurs in in the PSCs. As the temperature of the PSCs were gradually raised there was evidence of a progressive distortion of the packing of the chains from orthorhombic or monoclinic to near-hexagonal up to the Tp (in 14:0-PSC) or the Tm (in 16:0-PSC and 18:0-PSC). Only 14:0-PSC gave clear evidence of a pretransition in the gel state in full agreement with the calorimetric studies. However, it was unclear whether a pretransition occurred in 16:0-PSC or 18:0 PSC because of the large influence of the Tm on the spectra. The crystal packing of the chains of 14:0-PSC between Tp and Tm are thought to be hexagonal or triclinic. Further studies by X-ray diffraction are required to assign unequivocably the proper crystal packing of the acyl chains in the gel state. Another point that would be worthwhile investigating is whether the PSC homologues with lauroyl chains or shorter would exhibit a Tp at temperatures further removed from Tm.

As was observed for PC, the bands for the head group of PSC proved to be insensitive to temperature and phase changes. In lecithin this insensitivity is thought to be due to the large hydration sphere bound to the charged polar head moiety (80). This explanation may apply to the less hydrated polar head group in PSC.

DPPSC exhibits a subtransition in the same way as DPPC except that it is slower to occur and the onset of Ts occurs 2 °C higher in DPPSC than in DPPC. This Ts is thought to be due to repacking of the acyl chains at these low temperatures.

2.6.5 Nuclear Magnetic Resonance

The NMR study has confirmed that D_3-DPPSC (labelled in the head methyl) behaves very much like the analogous DPPC in aqueous dispersions. Both the ^{31}P and the ^{2}H NMR spectra (Figs 35 & 37) of D_3-DPPSC are consistent with the multilamellar structures as those for the PC species as expected from the freeze fracture E.M micrographs (Fig. 28). The identical values for the quadrupole splitting for D_3-DPPSC and for D_9-DPPC, at the same temperatures, do not support the hypothesis presented above (section 2.6.1) that the higher transition temperature of PSC is due to an increase in the electrostatic interaction between the positively charged region of one molecule and the negatively charge phosphate residue of a neighboring molecule. The quadrupole splitting may not be sufficiently sensitive to detect the relatively small changes in polar head interaction expected for the small difference observed in Tm's. Since QS is dependent on both the rate and the amplitude of motion of the head group, the variation of one parameter may be cancelled out by the other. In any case, it was not possible to separate the individual motional contributions because of the lack of information on the geometry and motional freedom of the head group. These questions may be answered by X-ray diffraction studies where it was found that the orientation of the head group of PC is almost parallel to the plane of the bilayer in both the gel and fluid states (132). The similarity in the QS values between D_3-DPPSC and D_9-DPPC may be indicative of a similar orientation of the two head groups.

The variation of the first and second moments of the ^{2}H-NMR spectra of D-3 DPPSC, over the temperature range 0-20 °C, is suggestive of increasing rates of molecular motion and decreasing molecular ordering with increasing temperature. The plateaus in the temperature dependence curves

(Fig. 36) of these parameters between 20-40 °C is quite different from the reported monotonic decrease seen for perdeuterated DPPC (100). The side chain and the head group then appear to behave somewhat independently at these temperatures. Finally, the "Jarrell calculation " (164) using the first three moments indicates that the head group of PSC experiences phase transition effects 2°C below the beginning of the phase transition as measured by calorimetry. This effect may be related to the reorganisation of the acyl chains, from an orthorhombic to a hexagonal packing as suggested by FTIR (section 2.6.4).

Finally, the transition temperature (45 °C) of the deuterated derivative as measured from the first and second moments of the ^{2}H-NMR peaks (Fig. 36) and from the residual chemical shift of the ^{31}P-NMR (Fig. 38) is in agreement with the value obtained by calorimetry.

2.6.6 Monolayer Studies

The fact that, the minimum cross-sectional areas (Fig. 45) of the 18:0 PSC and 18:0-PC were identical, indicates that the larger head group of PSC does not cause steric hindrance in the tight packing of the stearic acid chains at the point just prior to the collapse of the monolayer. However, the higher surface tension of the PSC monolayer compared to the PC monolayer, in the intermediate area where the polar heads are said to interact, is an indication that the PC head group is more highly hydrated than the PSC head group. This is consistent with the qualitative observation that the quantity of water hydrated by PC bilayers is greater than that hydrated by PSC bilayers. In contrast, the polar head of PSC in non aggregated form, in the expanded film, appears to be slightly more hydrated than the corresponding PC. The phase transition from the gaseous to the liquid state demonstrated by the break in the compression curve of PSC monolayer and the lack of a transition in PC monolayer supports the thesis that the head groups of PSC are more interactive than the head groups of PC.

2.7 CONCLUSIONS

One might have predicted that the physical behaviour of PSCs compared to PC would have been affected by the presence of the sulfonium group to a greater extent than was actually observed. In fact, the properties common to both analogues, PC and PSC are numerous:

1. Similar polymorphic behaviour, e.g. formation of bilayer phase, multilamellar vesicles (MLVs), singlelamellar vesicles after sonication of MLVs and monolayers at air-water interface, but not hexagonal phase;
2. similar thermotropic behaviour, e.g. main transition temperatures, enthalpies and entropies of the gel to liquid crystalline transition, presence of a pretransition in one PSC (di14:0-PSC), presence of a subtransition under special conditions;
3. similar bilayer properties, e.g. similar dynamic order at the center of the bilayer, similar crystal lattice structure in the gel state in the saturated derivatives, similar mobility of the head group of the 16:0- analogues, similar minimum cross-sectional area of 40 Å for the saturated analogues (measured in the monolayer);
4. similar interaction with cholesterol, e.g. the abolishment of the gel to liquid crystalline transition of di-16:0 analogues at a mole ratio of 1:1, similar permeability properties of 14:0 analogues in the presence of cholesterol (published separately) (112).

It would appear then that the hydrocarbon chain interaction of PSC is not appreciably influenced by its having a larger polar head group than in PC. This suggests that the PSC group may be tilted differently with respect to the plane of the hydrocarbon chains than the PC group. There are differences between these two analogues that may be attributed to the sulfonium head group:

1. The slightly higher Tm's (2-4 °C) in the saturated PSCs than in the corresponding PCs;
2. the lack of a Tp in di-16:0-PSC and di-18:0-PSC; the presence of a Tp in di-18:1-PSC; the higher and sharper Tp in di-14:0 PSC;
3. the greater hydration of the polar head of PCs than of PSCs;
4. the greater associative interaction of the polar heads of PSC;
5. the greater distance between the acyl chains of PSC in the region just below the ester bonds of the molecule in the bilayer;
6. the slightly greater permeability (10 %) of PSC bilayers to non-polar molecules like urea and carboxyfluorescein (published separately) (112).

These properties of PSCs are not very dissimilar from those of the PCs but tend toward the behaviour of PE. The phosphosulfocholine group of PSCs appear to be more interactive than the head group of PC which may be attributed to the tilt of the respective head groups of PSC and PC. Further studies by X-ray diffraction are required to establish the tilt of the polar head group of PSC relative to that of PC and to determine the packing of the acyl chains in the gel state.

The present findings of a general similarity in the physical properties of PC and PSC indicate that the sulfonium analogue should be able to substitute functionally for PC in natural membranes. Clearly such a functional substitution occurs in membranes of the marine diatom *Nitzschia alba* (1), in which PC is completely replaced by PSC. Recently, it has been shown that virtually complete replacement of choline by sulfocholine in phospholipids of LM cells (13) and of the yeast *Saccharomyces cerevisiae* (63) can be achieved with little change in cell growth. In general, analogues of PC in which the nitrogen is replaced by other elements of group Vb or VIb of the periodic table (e.g. P, As, S. etc) can substitute adequately for choline in the biosynthesis of PC in animal tissue (179). Finally, it would appear that any 'onium' polar head group (sulfonium, phosphonium, arsonium, etc.). should be able to substitute for the quaternary ammonium group in PC (180).

BIBLIOGRAPHY

1. Anderson, R., Kates, M., Volcani, B.E., Nature, 263 (1976) 51-53.

2. Ladbrooke, B.D., Chapman, D., Chem. Phys. Lipids, 3 (1969) 304-356.

3. Kates, M., Tremblay, P.-A., Anderson, R., Volcani, B.E., Lipids, 13 (1978) 34-41.

4. Kates, M., Volcani, B.E., Biochim. Biophys. Acta, 116 (1966) 264-278.

5. Anderson, R., Livermore, B.P., Kates, M., Volcani, B.E., Biochim. Biophys. Acta, 528 (1978) 77-88.

6. Anderson, R., Kates, M., Volcani, B.E., Biochim. Biophys. Acta, 523 (1978) 89-106.

7. Harwood, J.L., in *The Biology of Plants*, 4 (1980) 301-320, (Stumpf, P.K., ed.), Academic Press Inc, New York.

8. Karpyshev, N.N., Bushnev, A.S., Zvonkova, E.N., Evstigneeva, R.P., Bnooptahnyeckar XNMNR, 3 (1977) 1374-1377.

9. Tornabene, T.G., Kates, M., Volcani, B.E., Lipids, 9 (1974) 279-284.

10. Rubinstein, I., Goad, L.J., Phytochemistry, 13 (1974) 485-487.

11. Goad, L.J., Lenton, J.R., Knapp, F.F., Goodwin, T.W., Lipids, 9 (1974) 582-596.

12. Anderson, R., Kates, M., Volcani, B.E., Biochim. Biophys. Acta, 573 (1979) 557-561.

13. Anderson, R., Bilan, P., Biochim. Biophys. Acta, 640 (1981) 91-96.

14. Bjerve, K.S., Bremer, J., Biochim. Biophys. Acta, 176 (1969) 570-583.

15. Kates, M., in *Methods in Membrane Biology*, (Korn, E.D., ed.), 8 (1977) 219-290, Plenum Publishing, New York.

16. Rosenthal, A.F., Methods in Enzymology, 35 (1975) 429-529.

17. Slotboom, A.J., Verheij, H.M., de Haas, G.H., Chem. Phys. Lipids, 11 (1973) 295-317.

18. Jensen, R.G., Topics in Lipid Research, 3 (1972) 1-35.

19. Slotboom, A.J., Bonsen, P.P.M., Chem. Phys. Lipids, 5 (1970) 301-398.

20. Baer, E., Kates, M., J. Am. Chem. Soc., 72 (1950) 942-949.

21. Baer, E., Maurukas, J., J. Am. Chem. Soc., 74 (1952) 158-160.

22. Baer, E., Buchnea, D., Newcombe, A.G., J. Am. Chem. Soc., 78 (1956) 232-237.

23. Hirt, R., Berchtold, R., Pharm. Acta Helv., 33 (1958) 349-356.

24. Eible, H., Arnold, D., Weltzien, H.V., Westphal, O., Liebigs Ann. Chem., 709 (1967) 226-230.

25. Baer, E., Kindler, A., Biochemistry, 1 (1962) 518-521.

26. Tattrie, N.H., McArthur, C.S., Can. J. Biochem. Physiol., 35 (1957) 1165-1170.

27. Kogl, F., de Haas, G.H., van Deenen, L.L.M., Rec. Trav. Chim., 79 (1960) 661-674.

28. Baer, E., Buchnea, D., Can. J. Biochem. Physiol., 37 (1959) 953-959.

29. Cubero Robles, E., van den Berg, D., Biochim. Biophys. Acta, 187 (1969) 520-526.

30. Pugh, E., Kates, M., Journal of Lipid Research, 16 (1975) 392-395.

31. Gordon, D.T., Jensen, R.G., Lipids, 7 (1972) 261-262.

32. Brockerhoff, H., Yurkowski, M., Can. J. Biochem., 43 (1965) 1777.

33. Chadha, J.S., Chem. Phys. Lipids, 4 (1970) 104-108.

34. Cubero Robles, E., Roels, G.F.M., Chem. Phys. Lipids, 6 (1971) 31-38.

35. Tattrie, N.H., McArthur, C.S., Biochem Prep., 6 (1958) 16-19.

36. Warner, T.G., Benson, A.A., Journal of Lipid Research, 18 (1977) 548-552.

37. Patel, K.M., Morissett, J.D., Sparrow, J.T., Journal of Lipid Research, 20 (1979) 674-677.

38. Aneja, R., Chadha, J.S., Biochim. Biophys. Acta, 248 (1971) 455-457.

39. de Haas, G.H., van Deenen, L.L.M., Rec. Trav. Chim., 80 (1961) 951-970.

40. de Haas, G.H., van Deenen, L.L.M., Tetrahedron Letters, 22 (1960) 7-11.

41. Bird, P.R., de Haas, G.H., Heemskerk, C.H.T., van Deenen, L.L.M., Biochim. Biophys. Acta, 98 (1965) 566-573.

42. Tinoco, J., McIntosh, D.J., Chem. Phys. Lipids, 4 (1970) 72-84.

43. Ghosh, D., Lyman, R.L., Tinoco, J., Chem. Phys. Lipids, 7 (1971) 173-184.

44. Ghosh, D., Tinoco J., Biochim. Biophys. Acta, 266 (1972) 41-49.

45. Demel, R.A., Geurts van Kessel, W.S.M., van Deenen, L.L.M., Biochim. Biophys. Acta, 266 (1972) 26-40.

46. Renshaw, R.R., Bacon, N., Roblyer, J.H., J. Am. Chem. Soc., 45 (1926) 517-520.

47. Maw, G.A., du Vigneaud, V.J., Journal of Biological Chemistry, 176 (1948) 1029-1045.

48. Baer, E., Journal of Biological Chemistry, 189 (1951) 235-247.

49. Uhlenbroek, J.H., Verkade, P.E., Rec. Trav. Chim., 72 (1953) 395-410.

62. Hessel, L.W., Morton, I.D., Todd, A.R., Verkade, P.E., Rec. Trav. Chim., 73 (1954) 150-156.

51. Stanacev, N.Z., Kates, M., Can. J. Biochem. Physiol. 38 (1960) 297-300.

52. van Deenen, L.L.M., de Haas, G.H., Advances in Lipid Research, 2 (1964) 167-234.

53. Baer E., Buchnea, D., Arch. Biochem. Biophys., 78 (1958) 294-305.

55. Bonsen, P.P., de Haas, G.H., Chem. Phys. Lipids, 1 (1967) 100-109.

56. Lapidot, Y., Barzilay, I., Hajdu, J., Chem. Phys. Lipids, 3 (1969) 125-134.

57. Kates, M., Can. J. Biochem. Physiol., 33 (1955) 575-589.

58. Faure, M., Troestler, J., Bull. Soc. Chim. Biol., 49 (1967) 279-290.

59. Kates, M., Sastry, P.S., Methods in Enzymology, 14 (1969) 197-208.

60. Vogel, A. I., in *Practical Organic Chemistry*, 3rd edition, 1956, Longmans, Green and Co., London, New York.

61. Fieser, L.F., and Fieser, M., in *Reagents for Organic Synthesis*, 1 (1972), John Wiley and Sons, inc., New York, London, Sydney.

63. Tremblay, P.-A., Kates, M., Chem. Phys. Lipids, in preparation, (1982).

64. Hanahan, D.J., Turner, M.B., Jayko, M.E., Journal of Biological Chemistry, 192 (1951) 623-628.

65. Kates, M., in *Laboratory Techniques in Biochemistry and Molecular Biology, Techniques in Lipidology*, 1972, (Work, T.S., Work E., eds), North Holland Publishing Co., Amsterdam, London.

66. Wagner, H., Horhammer L., Wolff, P., Biochem. Z., 334 (1961) 175-181.

67. Beiss, U., J. Chromatography, 13 (1964) 104-110.

68. Dittmer, J.C., Lester, R.L., Journal of Lipid Research, 5 (1964) 126-127.

69. Bligh, E.C., Dyer, W.T., Can. J. Biochem. Physiol., 37 (1959) 911-917.

70. De la Roche, A.I., Andrews, C.J., Kates, M., Plant Physiology, 51 (1973) 468-473.

71. Allen, R.J.L., Biochemical Journal, 34 (1940) 858-865.

72. Bartlett, G.R., Journal of Biological Chemistry, 234 (1959) 466-468.

73. Carroll K.K., Nature, 191 (1961) 377-378.

74. Baer, E., Biochemical Prep., 2 (1952) 31-38.

75. LeCocq, J., Ballou, C.E., Biochemistry, 3 (1964) 976-980.

76. Kates, M., Chan, T.H., Stanacev, N.Z., Biochemistry, 2 (1963) 394-397.

77. Sowden, J.C., Fisher, H.O.L., J. Am. Chem. Soc., 63 (1941) 3244-3248.

78. Woese, C.R., Scientific American, 244 (1981) June, 98-122.

79. Brotherus, B., Renkonen, G., Chem. Phys. Lipids, 13 (1974) 178-183.

80. Casal, H., Phd. Thesis, University of Ottawa (1981).

81. Wood, G.W., Law. P.-Y., Morrow, G., Rao, G.W.S., Schmidt, J.O.E., Tuebner, J., Chem. Phys. Lipids, 18 (1977) 316-333.

82. Wood, G.W., Law, P.-Y., Rao, G.N.S., Biomed. Mass Spectrom., 3 (1976) 172-176.

83. Kates, M., Pugh, E.L., in Membrane Fluidity (1980) 153-170, (Kates, M., Kuksis, A., eds.) Humana Press, Clifton, N.J.

84. Snyder, F., Lee, T.C., Blank, M.L., Moore, C., in Membrane Fluidity (1980) 307-326, (Kates, M., Kuksis, A., eds) Clifton, N.J.

85. Akesson, B., in Membrane Fluidity (1980) 419-434, (Kates, M., Kuksis, A., eds) Clifton, N.J.

86. Israelachvili, J.N., Mitchell, D.J., Minham, B.W., Biochim. Biophys. Acta, 470 (1977) 185-201.

87. Boggs, J.M., Can. J. Biochem., 58 (1980) 755-770.

88. Tanford, C., in The Hydrophobic Effect: Formation of Micelles and Biological Membranes, (1972) Wiley Interscience Publications, New York.

89. Chapman, D., Q. Rev. Biophys., 8 (1976) 185-235.

90. Shipley, G.G., in Biological Membranes, 2 (1973) 1-89, (Chapman, D., Wallach, D.F.H., eds), Academic Press, N.Y.

91. Lee, A.G., Prog. Biophys. Mol. Bio., 29 (1975) 3-56.

92. Gaines, G.L., in Insoluble monolayers at Liquid Interfaces, (1966) Wiley-Interscience, New York.

93. Shah, D.O., Progr. Surf. Sci., 3 (1972) 221-278.

94. Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L., Thompson, T.E., Biochemistry, 15 (1976) 1393-1401.

95. Papahadjopoulos, D., Kimelberg, J.K., Prog. Surf. Sci., 4 (1975) 141-232.

96. Tinker, D.O., Pinteric, L., Biochemistry, 10 (1971) 860-865.

97. Hildebrand, J.H., Proc. Natl. Acad. Sci., U.S.A., 69 (1972) 3428-3431.

98. Belanger, Y., PhD. Thesis, University of Ottawa, 1980.

99. Seelig, A., Seelig, J., Biochemistry, 13 (1974) 4839-4845.

100. Davis, J.H., Biochemical Journal, 27 (1979) 339-358.

101. Cameron, D.G., Casal, H.L., Mantsch, H.H., Biochemistry, 19 (1980) 3665-3672.

102. Cameron, D.G., Casal, H.L., Gudgin, E.F., Mantsch, H.H., Biochim. Biophys. Acta, 596 (1980) 463-467.

103. Cameron, D.G., Casal, H.L., Mantsch, H.H., Journal Biochem. Biophys. Methods, 1 (1979) 21-36.

104. Kornberg, R.M., McConnell, H.M., Biochemistry, 10 (1971) 1111-1120.

105. Brulet, P., McConnell, H.M., Proc. Natl. Acad. Sci., U.S.A., 72 (1975) 1451-1455.

106. Devaux, P., McConnell, H.M., Ann. N.Y. Acad. Sci., 222 (1974) 489-498.

107. Levine, Y.K., Birdsaal, N.J.M., Lee, A.G., Metcalfe, J.C., Biochemistry, 11 (1972) 1416-1421.

108. Schinder, H., Seelig, J., Journal Chemical Physics, 59 (1973) 1841-1850.

109. Gaffney, B.J., Chen, S.-C., in Methods in Membrane Biology, 8 (1977) 291-368, (Korn, E.D., ed.).

110. Devaux, P., Bienvenue, A., Lauquin, G., Brisson, A.D., Vignais, P.M., Vignais, P.V., Biochemistry, 14 (1975) 1272-1280.

111. Roseman, M., Litman, B.J., Thompson, T.E., Biochemistry, 14 (1975) 4826-4830.

112. Bittman, R., Leventhal, A.M., Karp, S., Blau, L., Tremblay, P.-A., Kates, M., Chem. Phys. Lipids, 28 (1981) 323-335.

113. Lakowicz, J.R., Prendergast, F.G., Hogan, D., Biochemistry, 18 (1979) 508-519.

114. Janiak, M.J., Small, D.M., Shipley, G.G., Journal of Biological Chemistry, 254 (1979) 6088-6098.

115. Janiak, M.J., Small, D.M., Shipley, G.G., Biochemistry, 15 (1976) 4575-4580.

116. Keough K.M.W., Biochemistry, 18 (1979) 1453-1459.

117. Hinz, H.J., Sturtevant, J.M., Journal of Biological Chemistry, 247 (1972) 6071-6075.

118. Lands, G., in <u>Membrane Fluidity</u>, (1980) 419-434, (Kates, M., Kuksis, A., eds) Humana Press, Clifton N.J.

119. Liu, N.-I., Kay, R.I., Biochemistry, 16 (1977) 3484-3486.

120. Chen, S.C., Sturtevant, J.M., Gaffner, B.J., Proc. Natl. Acad. Sci., U.S.A., 77 (1980) 5060-5063.

121. Mantsch, H.H., personal communication, 1981.

122. Cullis, P.R., de Kruijff, B., Biochim. Biophys. Acta, 513 (1978) 31-42.

123. Junger, E., Reinauer, H., Biochim. Biophys. Acta, 183 (1969) 304-342.

124. Rand, R.P, Tinker, D.O. Fast, P.G., Chem. Phys. Lipids, 6 (1971) 333-342.

125. Phillips, M.C., Chapman, D., Biochim. Biophys. Acta, 163 (1968) 301-313.

126. Hauser, H., Phillips, M.C., Levine, B.A., Williams, R.J.P., Nature, 261 (1976) 390-394.

127. Hitchcock, P.B., Mason, R., Thomas, K.M., Shipley, G.G., Proc. Natl. Acad. Sci., U.S.A., 71 (1974) 3036-3040.

128. Seelig, J., Gally, H.U., Wohlgemuth, R., Biochim. Biophys. Acta, 467 (1977) 109-117.

129. Papahadjopoulos, D., Biochim. Biophys. Acta, 163 (1968) 240-254.

130. Papahadjopoulos, D., Weiss, L., Biochim. Biophys. Acta, 183 (1969) 417-426.

131. Eibl, H., in Polyunsaturated Fatty Acids, (1977) 229-244, (Kuman, W.H., Holman, R.T., eds) AOCS, Champain, Il.

132. Bûldt, G., Wohlgemuth, R., Journal in Membrane Biology, 58 (1981) 81-100.

133. Michaelson, D.M., Hoiwitz, A.F., Klein, M.P., Biochemistry, 13 (1974) 2605-2612.

134. Jendrasiak, G.L., Hasty, J.H., Biochim. Biophys. Acta, 337 (1974) 79-91.

135. Macdonald, R.C., Simon, S.A., Baer, E., Biochemistry, 15 (1976) 885-891.

136. Hsia, J.C., Boggs, J.M., Biochim. Biophys. Acta, 266 (1972) 18-25.

137. Haydon, D.A., Taylor, J.L., J. Theoret. Biol., 4 (1963) 281-296.

138. Tokutomi, S., Ohki, K., Ohnishi, S.-I., Biochim. Biophys. Acta, 596 (1980) 192-200.

139. Abramson, M.B., Calacicco, G., Curci, R., Rapport, M.M., Biochemistry, 7 (1968) 1692-1698.

140. Cullis, R., Can. J. Biochem., 26 (1981) 89-96.

141. Jain, M.K., Curr. Topics in Membrane Transport, 6 (1975) 1-57.

142. de Kruijff, B., Demel, R.A., van Deenen, L.L.M., Biochim. Biophys. Acta, 255 (1972) 331-347.

143. Umemura, J., Cameron, D.G., Mantsch, H.H., Biochim. Biophys. Acta, 602 (1980) 32-44.

144. Singer, S.J., Nicholson, G.L., Science, 175 (1972) 720-731.

145. Cullis, P.R., de Kruijff, B., Biochim. Biophys. Acta, 559 (1979) 399-420.

146. Parker, C.A., in Photoluminescence of Solutions (1968) 51-65, Elsevier press, New York.

147. Berlman, I.B., in Handbook of Fluorescense Spectra of Aromatic Molecules, (1965) Academic Press, New York.

148. Radda, G.K., in Fluorescent Probes in Membrane Studies, in Methods in Membrane Biology, 4 (1975) 97-188, (Korn, E.D., ed.).

149. Gilmore, R., Cohn, N., Glaser, M., Biochemistry, 18 (1979) 1042-1049.

150. Shinitzky, M., Inbar, M., Biochim. Biophys. Acta, 433 (1976) 133-149.

151. Chen, L.A., Dale, R.E., Roth, S., Brand, L., Journal of Biological Chemistry, 252 (1977) 2163-2169.

152. Sklar, L.A. Phd. Thesis, 1978.

153. Sklar, L.A., Hudson, B.S., Petersen, M., Diamond, J., Biochemistry, 16 (1977) 813-818.

154. Sklar, L.A., Hudson, B.S., Petersen, M., Diamond, J., Biochemistry, 16 (1977) 819-828.

155. Hubbell, W.L., McConnell, H.M., Proc. Natl. Acad. Sci. of U.S.A., 61 (1968) 12-24.

156. Hubbell, W.L., McConnell, H.M., Proc. Natl. Acad. Sci. of U.S.A., 63 (1969) 645-652.

157. Hubbell, W.L., McConnell, H.M., Proc. Natl. Acad. Sci. of U.S.A., 63 (1969) 20-28.

158. Libertini, L.J., Waggonner, A.S., Jost, P.C., Griffith, O.H., Proc. Natl. Acad. Sci. of U.S.A., 64 (1969) 13-18.

159. McConnell, H.M., Gaffney-McFarland, B., Ann. N.Y. Acad. Sci., 195 (1972) 207-214.

160. Libertini, L.J., Burke, C.A., Jost, P.C., Griffith, O.H., J. Magn. Res. 15 (1974) 460-467.

161. Gaffney, B.J., McConnell, H.M., J. Magn. Res., 16 (1974) 1-8.

162. Schreier-Muccillo, S., Smith, I.C.P., Prog. Surf. Memb. Sci., 9 (1973) 1-34.

163. Rance, M., Jeffrey, K.R., Tulloch, A.P., Butler, K.W., Smith, I.C.P., Biochim. Biophys. Acta, 600 (1980) 245-262.

164. Jarrell, H.C., Byrd, R.A., Smith, I.C.P., Biophys. J., in press.

165. Bloom, M., Davis, J.H., Dahlquist, F.W., in Proceeding of the 20th Ampere Congress, Tallin, Estonia, (1978) 551, (Kundla, E., Lippman, E., Saluvere, T., eds.) Springer Verlag, Berlin.

166. Byrd, R.A., Smith, I.C.P., Tremblay, P.-A., Kates, M., Can. J. Biochem., 26 (1981) 84-88.

167. Verkleij, A.S., Vervegaert, P.H.D., van Deenen, L.L.M., Elbers, P.J., Biochim. Biophys. Acta, 288 (1972) 326-332.

168. Hubbell, W.L., McConnell, H.M., J. Am. Chem. Soc., 93 (1971) 314-326.

169. Stuhne-Stekalec, L., Stanacev, N.Z., Can. J. Biochem., 55 (1977) 173-185.

170. Schreier, S., Polnaszek, C.F., Smith, I.C.P., Biochim. Biophys. Acta, 515 (1978) 375-436.

171. Butler, K.W., Tulloch, A.P., Davis, J.H., Smith, I.C.P., FEBS Lett., 100 (1979) 57-61.

172. Spiess, H.W., in NMR Basic Principles and Progress, (1978) 55-214, (Diehl, P., Fluck, E., Kosfield, R., eds), Springer Verlag, Berlin.

173. Skarjeine, R., Oldfield, E., Biochemistry, 18 (1979) 5903-5909.

174. Stockton, G.W., Polnaszek, C.F., Leitch, L.C., Tulloch, A.P., Smith, I.C.P., Biochem. Biophys. Res. Comm., 60 (1974) 844-850.

175. Stockton, G.W., Polnaszek, C.F., Tulloch, A.P., Hasan, F., Smith, I.C.P., Bio, 15 (1976) 954-966.

176. Galley, H.-U., Niederberger, W., Seelig, J., Biochemistry, 14 (1975) 3647-3652.

177. Seelig, J., Biochim. Biophys. Acta, 515 (1978) 105-140.

178. Sheppard, N., in Advances in Spectroscopy, 1 (1959) 288-353, (Thompson, H.W. ed.) Interscience Inc.

179. Edwards, R.G., Hands, A.R., Biochim. Biophys. Acta, 431 (1976) 303-316.

180. Sim, E., Cullis, P.R., Richards, R.E., Biochemical Journal, 151 (1975) 555-560.

181. Zingsheim, H.P., Plattner, H., in Methods in Membrane Biology, 7 (1976) 1-146.

182. Pauling, L., in The Nature of the Chemical Bond, (1945) 167-189, Cornell University Press, Ithica, New York.

183. Kauppinen, J.K., Moffat, D.J., Mantsch, H.H., Cameron, D.G., Applied Spectroscopy, notes no. 38, may/june 1981.

184. Fuldner, H.H., Biochemistry, 20 (1981) 5707-5710

185. Fuldner, H.H., (1980), Ph.D. Thesis, Universitat Ulm, Ulm, FRG.

186. Taylor, M.G., Smith, I.C.P., Biochemistry, 20 (1981) 5252-5255.

187. Berde, C.B., Anderson, H.C., Hudson, B.S., Biochemistry, 19 (1980) 4279-4293.

188. Rand, R.P., Parsegian, V.A., Henry, J.A.C., Lis, L.J., McAllister, M., Can J. Biochem 59 (1980) 959-968.

189. Melchior, D.L., Scavillo, F.J., Stein, J.M., Biochemistry, 19 (1980) 4828-4834.

190. Mabrey, S., Mateom, P.L., Sturtevant, J.M., Biochemistry, 17 (1978) 2464-2468.

191. Lindbloom, G., Johansson, L.B.-A., Arvidson, G., Biochemistry, 20 (1981) 2205-2207.

192. Petri, W.A., Pal, R., Barenholz, Y., Wagner, R.R., Biochemistry, 20 (1981) 2796-2800.

193. Brainard, J.R., Cordes, E.H., Biochemistry, 20 (1981) 4607-4617.