THERMAL PROPERTIES OF LYSOPHOSPHATIDYLETHANOLAMINES

by

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INTRODUCTION:

One of the most important cellular organizations is the cell membrane. The detailed structure of the membrane recently has begun to be revealed, although the exact mechanism of fluidity control still eludes biochemists.

Past research has indicated that the membrane is composed of a bimolecular layer of phospholipids with protein, sphingolipids, and cholesterol interspersed throughout the structure. By employing phospholipid liposomes as model membrane systems, it has been observed that the fluid effect of the membrane is influenced by the type of fatty acids esterified to the phospholipids and by the amount of cholesterol present. However, the exact controlling mechanism of fatty acid composition and the amount of cholesterol present still remains a mystery.

Since the discovery that the membrane consisted primarily of diacyl phospholipids, countless papers have appeared dealing with the thermal properties of these compounds. The physical properties reported for these compounds have enabled many conclusions to be drawn with respect to the presence of two hydrocarbon chains on the phospholipid molecules.

Little work has been done regarding the absence of a hydrocarbon chain from the second carbon of the glycerol molecule of the phospholipid. In this study, the physical properties of lysophosphatidylethanolamines are examined with differential scanning calorimetry. The interactions of these compounds with cholesterol are also observed and comparisons of these results with results of similar studies on phosphatidylethanolamines, phosphatidylcholines, and lysophosphatidyl-

cholines are made to better understand fatty acid and cholesterol effects on membrane fluidity.

ABBREVIATIONS:

Some common abbreviations used throughout this thesis are the following:

PC - phosphatidylcholine

PE - phosphatidylethanolamine

LPC - lysophosphatidylcholine

LPE - lysophosphatidylethanolamine

DLPE - dilauroylphosphatidylethanolamine

DMPE - dimyristoylphosphatidylethanolamine

DPPE - dipalmitoylphosphatidylethanolamine

DSPE - distearoylphosphatidylethanolamine

DOPE - dioleoylphosphatidylethanolamine

LLPE - lauroyllysophosphatidylethanolamine

MLPE - myristoyllysophosphatidylethanolamine

PLPE - palmitoyllysophosphatidylethanolamine

SLPE - stearoyllysophosphatidylethanolamine

OLPE - oleoyllysophosphatidylethanolamine

^{31}PNMR - phosphorus nuclear magnetic resonance

^{2}HNMR - deuterium nuclear magnetic resonance

ESR - electron spin resonance

DSC - differential scanning calorimetry

sn - stereospecific numbering system of glycerol

LITERATURE REVIEW:

The structure and physiological function of the cell membrane has long been the focal point of considerable research in the field of biochemistry. In 1899, Overton (1) observed that the cell surface was impregnated with lipids, specifically lecithin and cholesterol, and that the rate of incorporation of any compound into a cell was directly proportional to the degree of "lipid solubility" of that compound.

Membrane Models and Hypotheses

Since Overton's work, membrane models and hypotheses have periodically appeared in the literature. In 1925, based on studies of lipids extracted from red blood cells, Gorter and Grendel (2) proposed that the membrane was a complete bimolecular lipid coat. Subsequent studies of actual membranes showed that natural membranes had lower interfacial tension than artificial membranes prepared from lipid extracts and thus, strongly suggested a requirement for protein at the membrane surface (3).

Upon obtaining indirect measurements of membrane thickness, birefringence, surface tension and other membrane properties, Danielli and Davson (4) in 1935 published their Classical Membrane Model. This hypothesis depicted the membrane as a bilayer of mixed lipids sandwiched between two layers of adsorbed protein (Figure 1A).

The Davson-Danielli membrane model remained undisputed until the late 1950's when Robertson (5) published the Unit Membrane Hypothesis. Robertson modified the Davson-Danielli model in that only one of the non-lipid layers surrounding

Figure 1A - The Davson-Danielli Membrane Model

Figure 1B - The Fluid Mosaic Membrane Model

(Drawings taken from Bloom and Fawcett, A Textbook of Histology, 10^{th} Ed., W.B. Saunders, Philadelphia, PA, 1975.)

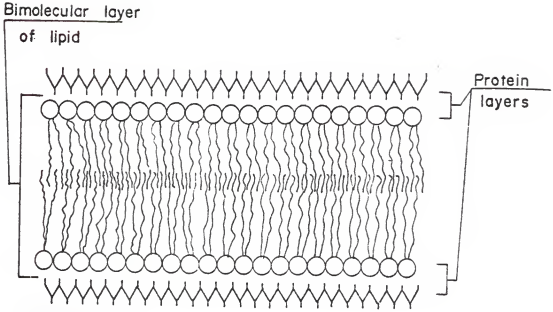


FIGURE 1 A

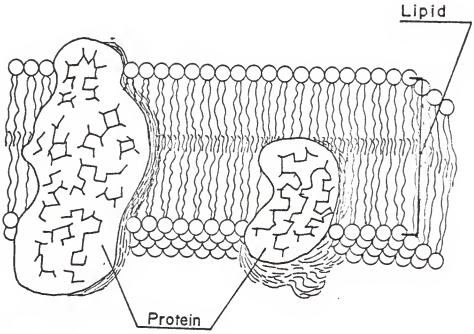


FIGURE 1 B

the lipid layer had to be protein and that the number of lipid layers in the membrane was limited to a single bimolecular layer, as evidenced by the 75 Å OsO_4 and $KMnO_4$ fixed electron micrograph bands of numerous membranes. Since this same basic structure appeared in most organelle and cell preparations, the hypothesis was called the "Unit Membrane" model.

However, the membrane model that is accepted most widely today is Singer and Nicholson's (6) Fluid Mosaic Membrane Model (Figure 1B). In essence, this hypothesis proposes that the globular protein components of the membrane are partially or completely embedded in a fluid bimolecular phospholipid matrix. The membrane consists of two diacyl phospholipid monolayers with the hydrophobic hydrocarbon chains of each layer directed toward the interior of the membrane and the hydrophilic polar headgroups of the phospholipids aligned along the exterior surface of the bilayer.

At physiological temperatures, the hydrocarbon chains are in a highly mobile or fluid, liquid-crystalline state (7). In this state, the two chains do not have physically identical behavior (8). The hydrocarbon chains of both choline (9,10) and ethanolamine (11) diacyl phospholipids have approximately the same deuterium magnetic resonances and for the most part are oriented perpendicular to the surface normal of the bilayer. However, the first few methylene groups of the hydrocarbon chain esterified at the second glycerol carbon is oriented parallel to the bilayer normal and then bends perpendicular to the normal and follows the first chain away from the bilayer surface and polar headgroups (9-11).

From deuterium magnetic resonance studies, the polar group has been considered to parallel the bilayer normal (12-14) with the phosphate of one headgroup interacting with the choline portion of an adjacent headgroup(s), as evidenced by a substantial ^{31}P nuclear Overhauser effect (14). However, the conformation and motion of the choline headgroup is extremely temperature dependent and its orientation is not fixed (13). The results of ^{31}P and ^{2}H NMR studies have indicated that increasing the temperature of a phospholipid system rotated the choline methyl groups in the vicinity of the phosphate group (13).

Cholesterol is also a major constituent of membranes and is found there at various concentrations. For example, in red blood cells and myelin membranes, cholesterol is asymmetrically distributed throughout the membranes and is present at approximately 40 mole % (15).

The cyclopentanoperhydrophenanthrene ring system of cholesterol extends into the inner hydrocarbon chains of the bilayer (15-17). The cholesterol molecule does not exert the same effect upon all the protons along the hydrocarbon chains of the phospholipids (18). Using NMR (19) and ESR (20,21), it has been detected that cholesterol exerts an effect on the first ten methylene groups of the acyl chains so as to reduce their mobility. The last few methylene groups, depending on the number of carbon atoms present in the fatty acyl residue, and the terminal methyl groups are hardly affected by the cholesterol molecule (18,22).

X-ray and neutron diffraction studies have shown that

the hydroxy group of the cholesterol molecule is situated between the phospholipid polar groups (16,17,23). However, although the cholesterol molecule has a substantial effect on the phospholipid hydrocarbon chains, ^{31}P and ^{2}H NMR results have indicated that the steroid has little effect on the conformation of the polar headgroup (24).

It has been well established that the mammalian membrane is of such complexity that it is extremely difficult to study all the effects of the different constituents simultaneously. Consequently, several membrane model systems have been used to study membranes such as phospholipid monolayer studies (2,25, 26) and "black lipid membrane" bilayer studies (27,28).

The methods which have yielded the most information about the membrane originated from a procedure presented by Mueller et al (29) in 1962. Mueller and his associates published one of the first methods for forming 60-90-Å thick "membranes" from lipids extracted from red blood cells. This discovery lead to Bangham's procedure in 1965 for preparation of multilamellar liposomes (30) and it enabled Huang in 1969 to develop a method for the preparation of single bilayer phospholipid vesicles (31). The latter two are the most widely used models in studying phospholipids for membrane applications.

Various methods of study have been applied to these phospholipid vesicles, including ^{2}H and ^{31}P nuclear magnetic resonance (8-10,14,18,24,32) and x-ray and neutron diffraction (16,17,23,37) techniques. Also, differential scanning calorimetry has been an important method for membrane study

and has contributed considerable information concerning membrane fluidity characteristics. Since the largest percentage of phospholipids in membranes consists of diacyl phospholipids, most of the work cited in the literature has been on those compounds.

The Gel-Liquid Crystal Transition

It has long been understood that diacyl phospholipids undergo a gel to liquid crystal thermotropic transition and differential scanning calorimeters, which have the capacity to detect these transitions, have been used to study those compounds (33).

Typical differential scanning calorimetry (DSC) scans of diacyl phosphatidylcholines have shown a pretransition located several degrees below that where the main gel to liquid-crystalline transition appears (33-36). Raman spectroscopy of diacyl phosphatidylcholine-water gels has indicated that from -180°C to slightly below the pretransition, no changes were observed for the hydrocarbon chains in the phospholipid spectra (37).

Thus, the appearance of a pretransition peak for a diacyl phospholipid is the first significant event of a DSC scan. It was first thought that the pretransition was due to a positional ordering of the polar headgroups (33,38,39). However, ^{2}H and ^{31}P NMR spectra have indicated that the calorimetric pretransition is not associated with a polar headgroup conformational change (13).

Subsequent research has suggested that the pretransition corresponds to a change in tilt of the hydrocarbon chains (40).

When diacyl phosphatidylcholines exist as a gel, the hydrocarbon chains are tilted 58° with respect to the bilayer normal (19,36,41). As the temperature increases, the appearance of a pretransition peak on a DSC scan is associated with a conversion of the tilted chains to perpendicularly oriented hydrocarbon chains (40;See Figure 2).

As the temperature increases above the pretransition temperature, a major peak is observed on the typical DSC scan. The main peak of a DSC scan has been attributed to the gel to liquid-crystalline phase transition involving a "melting" of the hydrocarbon chains.

After the pretransition, the fatty acyl chains are oriented perpendicular to the bilayer normal. From low-angle x-ray studies, the hydrocarbon chains exist as straight, rigid rods where the carbon to carbon bonds (C-C) of the hydrocarbon chains are in an all-trans or near-all-trans configuration (42,43).

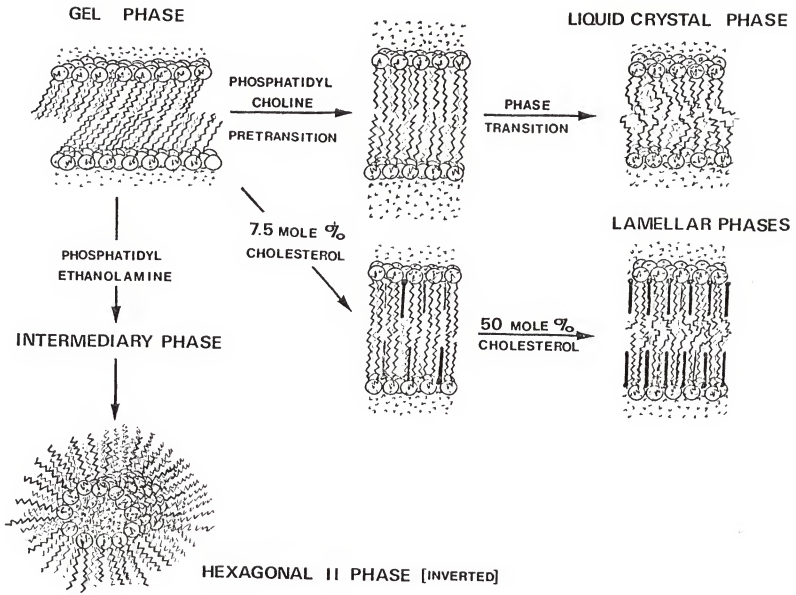
As the temperature increases and the transition appears, gauche conformations are introduced into the hydrocarbon chains (44). The gauche conformation is the result of a small partial rotation about the C-C bonds in the hydrocarbon chains of the phospholipid (45). Thus, "kinks" are introduced and the kinks permit changes in the directions of orientation of the fatty acyl chains and are depicted as a flexing or twisting of the acyl chains. Träuble and Sackmann have reported a diffusion coefficient for hydrocarbon chain kinks where $D_{diff} = 1 \times 10^{-5}$ cm^2/sec (42). This fast diffusion coefficient indicates the mobility of the gauche conformation.

Figure 2. Phase Transitions of Phospholipids.

The diacyl derivatives of both phosphatidylcholines and phosphatidylethanolamines exist in the gel phase at lower temperatures. However, the types of phases exhibited are different for PC as compared to PE.

The phosphatidylcholines undergo a pretransition which is associated with a straightening of the hydrocarbon chains. At this point, the hydrocarbon chains still exist as rigid rods. When the transition temperature is reached, trans-gauche isomerization occurs, the hydrocarbon chains become fluidized, and their direction of orientation becomes haphazard. For isothermal conditions, the addition of 7.5 mole % cholesterol converts the hydrocarbon chains from the tilted position to the vertical position and 50 mole % cholesterol sufficiently fluidizes the bilayers.

The phosphatidylethanolamines are converted to an intermediary phase upon a temperature increase and when the transition temperature is reached, the inverted hexagonal phase becomes the prominent structure.


GEL PHASE
LIQUID CRYSTAL PHASE
PHOSPHATIDYL CHOLINE
PRETRANSITION
PHASE TRANSITION
7.5 MOLE % CHOLESTEROL
LAMELLAR PHASES
PHOSPHATIDYL ETHANOLAMINE
INTERMEDIARY PHASE
50 MOLE % CHOLESTEROL
HEXAGONAL II PHASE [INVERTED]

The vibrational Raman spectra of dimyristoyl- (14:0), dipalmitoyl- (16:0), and distearoyl- (18:0) phosphatidylcholines above and below their corresponding transition temperatures have provided a method for detecting the relative frequencies of trans-gauche isomerization for phospholipid-water gels (43). From the spectra, Yellin and Levin (43) estimated the number of gauche bonds per phospholipid molecule at different temperatures. Below the transition temperatures of dimyristoyl- and dipalmitoylphosphatidylcholines, 2 gauche bonds per phospholipid molecule were calculated and for distearoylphosphatidylcholine, 6 gauche bonds were reported. As the temperature of the gels was increased to a point above the transition temperature, the number of gauche conformations per phospholipid molecule increased to 6 or 7, 8 or 9, and 13 or 14 respectively (43).

Nagle (46) proposed that when the gauche rotations occur, the bilayer has to expand and the bilayer thickness should decrease, which is what actually occurs. Träuble and Haynes (47) have compared volume changes of diacyl phospholipids, pure hydrocarbons, and palmitic acid and tripalmitin upon melting. Results indicate that the true melting of the hydrocarbon chains of the paraffin-like substances were very different than the melting of diacyl phospholipids, where the diacyl chains were still in a relatively ordered state as compared to the pure hydrocarbon substances (47). Thus, the main transition of diacyl phospholipids involves the formation of gauche rotations among the hydrocarbon chains and does not infer a true melting of the hydrocarbon chains.

The temperatures at which transitions occur are influenced by the polar headgroup and the type and number of fatty acyl residues present (33,49,50). Differential scanning calorimetry data of diacyl phospholipids have shown that the gel to liquid-crystalline transition temperature for a phosphatidylcholine with a pair of identical fatty acyl chains increases when an ethanolamine headgroup is substituted for the choline group (48). Also, for diacyl phosphatidylethanolamines with two identical hydrocarbon chains, the transition temperature is reduced by 7.7 to 8.7°C for each methyl group added to the ethanolamine headgroup (51). Consequently, large polar headgroups lead to lower transition temperatures. The nature of the fatty acid constituents also influence the transition temperature.

For diacyl phospholipids with identical polar groups, long hydrocarbon chains promote higher transition temperatures than the shorter ones (33) and saturated chains yield higher transition temperatures than the unsaturated ones (49). The nature of the unsaturation also has an effect on the transition temperature as unsaturated chains with trans configurations cause higher transition temperatures than those with cis configurations (50).

Differential scanning calorimetry provided a method for measuring the enthalpy of a thermotropic transition in addition to the temperature at which the transition occurs (33-35,48). Table I summarizes the results obtained to date. These data show that the transition enthalpy is generally proportional to the chain length of the constituent fatty acids.

Table I. Summary of Phospholipid Calorimetric Data

PHOSPHOLIPID	12:0		14:0		16:0		18:0		18:1		MOLE % CHOLESTEROL *	REFERENCE
	T_t^oC (A)	ΔH_t (B)	T_t^oC	ΔH_t	T_t^oC	ΔH_t	T_t^oC	ΔH_t	T_t^oC	ΔH_t		
DIACYL	0	-	23	6.64	41	8.66	58	10.67	-22	7.6	50	34
PHOSPHATIDYL	0	4.3	23	6.8	41.5	8.6	-	-	-14	11.2	33	48
CHOLINES	0	-	23	6.65	41	8.65	58	10.7	-22	7.6	-	33
	-	-	23.7	6.26	41.75	9.69	58.24	10.84	-	-	-	35
DIACYL	29	4.0	47.5	6.4	60	8.5	-	-	-16	4.5	33	48
PHOSPHATIDYL ETHANOLAMINES	-	-	49.5	5.8	-	-	-	-	-	-	-	82
LYSO	-43	2.3	-19	6.0	3	6.5	23	7.8	@	-	50	71
PHOSPHATIDYL CHOLINES	-	-	-	-	3.4	4.5	-	-	-	-	-	81

(A) T_t^oC - Temperature of Transition in ^oC

(B) ΔH_t - Enthalpy of Transition in Kcal/mole

* - Mole % Cholesterol Necessary to Eliminate a Thermal Transition

@ - No Thermal Transition Observed as low as -80^oC

Many authors reporting in the literature have noted an asymmetric distribution of phospholipids in naturally occurring membranes. Considerable work has been done on the red blood cell where 76% of the PC and 20% of the PE are on the outside of the membrane as compared to 24% and 80% of PC and PE respectively on the inside (52-54).

Since membranes are not made up of pure lipids, numerous studies on mixtures of phospholipids have appeared in the literature (55,56). DSC results on mixtures of two choline phospholipids with differences in fatty acyl chains of two methylene groups have shown that as the percentage of one phospholipid in the mixture was varied from 100% to 0%, a series of completely miscible gels was obtained. These gels produced DSC scans that were relatively sharp and cooperative at the extreme percentages and with somewhat broadened, less cooperative transitions at and around the 50%-50% mixture (56). Co-crystallization was observed for these types of phospholipid mixtures with no great variation in the melting range.

For phospholipids with the same species of hydrocarbon chains but different headgroup or for phospholipids with identical headgroups but different hydrocarbon chains, binary mixtures yielded a significantly increased melting range where clusters of gel and liquid-crystalline states appeared immiscible and lateral phase separations were observed, i.e. the two different phospholipids crystallized in separate regions (57).

It should be noted that the largest value of the heat of transition occurs with an equimolar aqueous dispersion of two phospholipids having different bases and where the difference

in hydrocarbon chain carbon number is greater than two methylene groups (55).

Cholesterol Effects Upon Phase Transitions

The addition of cholesterol to a pure phospholipid bilayer has also been studied to determine the effects on biomembranes. As early as 1966 it was reported that the addition of cholesterol to phospholipid at a mole ratio of 1:1 eliminated the major peak in the NMR spectra associated with the fatty acyl chains of the phospholipid (19).

Since 1966, considerable information has been published concerning the mechanisms of cholesterol interactions. As cholesterol is incorporated into phosphatidylcholine bilayers, the hydroxy group is aligned next to the phosphate group of the phospholipid (16,17,23). The cyclopentanoperhydrophenanthrene ring system of cholesterol aligns with the first ten methylene groups of the PC fatty acyl chains (19,21). ^{13}C NMR and ESR studies suggest that the "long-range swinging motion" of the hydrocarbon chains is substantially decreased upon the addition of cholesterol (58). However, the rate of rotational trans-gauche isomerization of the hydrocarbon chains is not significantly altered (58). Thus, at physiological temperatures, i.e. temperatures above the membrane phase transition, the number of gauche conformations is decreased upon the addition of cholesterol (24) and at cholesterol concentrations as high as 33 mole %, the bilayers remain in a fluid state (58).

The motion of the polar headgroup of the phosphatidylcholine bilayers is unaffected by the cholesterol content (58) and no conformational change is observed in the headgroup up-

on the addition of cholesterol as observed by ^{31}PNMR (24), even though the insertion of cholesterol separates the headgroups far enough apart to eliminate the intermolecular polar group interactions that were previously detected by nuclear Overhauser effects (59).

The overall effect of cholesterol addition to diacyl PC bilayers that are below the bilayer transition temperature increases the fluidity of the hydrocarbon chains and makes the bilayer more fluid. The presence of cholesterol above the transition temperature tends to stabilize the acyl chains and make them more rigid (60).

X-ray diffraction studies of phospholipid monolayers and liposomes have indicated that a secondary effect of the addition of cholesterol is a reduction of the average molecular areas of the phospholipid molecules and a comparable increase in bilayer thickness (23,61). This phenomenon coincides with the significant reduction in the hydrocarbon chain "long-range swinging motion" (58) in that when these motions are decreased, a tighter packing of the phospholipid molecules is allowed and a decrease in molecular area should result, which is what occurs (23,61).

Cholesterol does not interact with all phospholipids equally. In PC/PE mixtures, cholesterol has been found to be preferentially associated with the PC (48). However, cholesterol has an even greater affinity for sphingomyelin than for PC (62). Since the lipid composition of the human red blood cell has been shown to contain approximately 82% of the sphingomyelin, 76% of the PC, and 20% of the PE in the outer

layer and 18%, 24%, and 80% respectively and 100% of the phosphatidylserine in the inner layer (63), the above affinities suggest a reason why more cholesterol is found on the outside of the human red blood cell than on the inside (64).

Diacyl phosphatidylcholines form a gel below the transition temperature and upon the addition of cholesterol, transform into a neat lamellar phase (36; See Figure 2). Phosphatidylethanolamines in water are observed in a lamellar phase up to 20°C, transform into a mixture of the lamellar and inverted hexagonal phases over a wide concentration range, and at 55°C, the PE transforms to a complete inverted hexagonal phase (48,65-67; See Figure 2).

The addition of cholesterol to soya PE (18:1, 18:1) encouraged the formation of the hexagonal phase over a bilayer and the addition of unsaturated PC formed an intermediary phase instead of a bilayer (67). It was concluded that cholesterol destabilizes the bilayer formation in unsaturated lipid systems with PE which might explain why Escherichia coli and other gram negative bacteria have 85% unsaturated PE in the outer layer of their membranes (68) but have relatively low levels of cholesterol (62).

Demel et al reported that where cholesterol was found in high concentrations, PE was less than 20 mole %, i.e. erythrocyte, central nervous system, and peripheral nervous system membranes and where PE was found in mole percentages greater than 20 mole %, the cholesterol concentration was less than 15 mole %, i.e. mitochondrial, microsomal, and nuclear membranes (62).

Differential scanning calorimetry has been used to study cholesterol-phospholipid interactions. With increasing mole percentages of cholesterol, the diacyl phospholipid transition enthalpy and transition temperature are decreased linearly. For diacyl PC, both the 33 (35,41,69) and 50 (34,70) mole percentages of cholesterol have been reported to completely obliterate the thermotropic transition. For diacyl PE, 33 mole % cholesterol eliminates the thermotropic transition (48). No values of 50 mole % cholesterol have been reported for PE.

Lysophospholipids

All of this information has been accumulated for diacyl phospholipid-water gels and relatively little calorimetry work has been done on lysophospholipid-water dispersions. Klopfenstein has studied the effects of differing hydrocarbon chains and cholesterol interactions on the thermal properties of lysophosphatidylcholines (71). These results are summarized in Table I. Here, an inverse linear relationship was observed between mole percentages of cholesterol and transition enthalpy where 50 mole % cholesterol was sufficient to eliminate the DSC peak.

Analyses of membrane lipids have revealed that insignificant amounts of lysophospholipids are present in membranes and the main role of lysophospholipids has been attributed to metabolism and biosynthesis of diacyl phospholipids and cholesteryl esters (72). However, significant quantities of lysolipids have been discovered in the human lens (73). Broekhuyse analyzed the lipids of 20, 32, and 66 year old human lenses and the percentage of lysophospholipids of the total lipids

ranged from 9 to 20%, where LPE constituted the majority of the lysophospholipids (73).

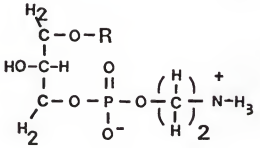
Several purification techniques of LPE have appeared in the literature (74,75) but nothing has been published describing any physical properties or DSC phenomena of these compounds.

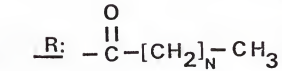
Since diacyl phosphatidylcholines, diacyl phosphatidylethanolamines, and lysophosphatidylcholines already have been studied by differential scanning calorimetry, the study of the calorimetric properties of lysophosphatidylethanolamines is the next logical step in order to better understand the effects the different polar headgroups, hydrocarbon chains, and cholesterol have on membrane structure and fluidity.

Figure 3. The Structure of Lysophosphatidylethanolamines.

The fatty acyl residues are esterified at the sn-1 position of the glycerol molecule, represented here as an R group. Common R groups esterified at sn-1 are lauric, myristic, palmitic, stearic, and oleic fatty acids. There is a tendency for Zwitterion formation in the aqueous systems with a negative charge on the phosphate oxygen and a positive charge on the ethanolamine quarternary nitrogen.

LYSOPHOSPHATIDYLETHANOLAMINES





12:0	LAURIC	N = 10
14:0	MYRISTIC	N = 12
16:0	PALMITIC	N = 14
18:0	STEARIC	N = 16
18:1	OLEIC	

MATERIAL AND METHODS:

The palmitoyllysophosphatidylethanolamine (PLPE), the oleoyllysophosphatidylethanolamine (OLPE), and the cholesterol were obtained from Serdary Research Laboratories (London, Ontario, Canada). The dilauroylphosphatidylethanolamine (DLPE) and the Eastern Cottonmouth snake venom (Ancistrodon piscivorus piscivorus) were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Lauroyllysophosphatidylethanolamine

Lauroyllysophosphatidylethanolamine (LLPE) was prepared by a phospholipase A_2 snake venom hydrolysis of the diacyl compound with Eastern Cottonmouth snake venom after Wykle and Snyder (76). 500 mg of DLPE were dissolved in 900 ml of anhydrous diethyl ether. 200 mg of the snake venom were dissolved in 25 ml of 2.5 mM calcium chloride in 0.1 M sodium borate buffer (pH = 7.0). This was added to the 900 ml ether. The reaction was allowed to proceed at room temperature for 6.5 hr at which time a sample was withdrawn for analysis by thin-layer chromatography.

Two silica gel plates were spotted with the reaction mixture and developed in the 65:25:4 chloroform/methanol/water solvent system with palmitoyllysophosphatidylethanolamine (PLPE) and dipalmitoylphosphatidylethanolamine (DPPE) as standards. Iodine vapors and 0.1% ninhydrin in acetone were used as color-developing reagents. The iodine vapors-developed plate indicated only two main spots corresponding to free fatty acids and lysophosphatidylethanolamine (LPE). The ninhydrin-developed plate indicated one positive spot corresponding to

the LPE and no traces of either the diacyl PE or glycero-PE compounds were present, indicating 100% conversion of the DLPE to LLPE.

The reaction mixture was then frozen at -9°C and thawed once to break the emulsion that formed. After a second freezing, the ether layer of the reaction mixture was poured off, taking a major portion of the crystallized LPE with it. The ether layer was taken to dryness on a rotary evaporator at room temperature and the contents were washed three times with 100 ml volumes of cold hexanes to remove free fatty acids. The residual hexane vapors were removed with the rotary evaporator and the crystallized LPE was dissolved in 40 ml of cold chloroform/methanol (2:1, v/v). The solution was divided equally by weight between two SS-34 Sorvall centrifuge tubes and were spun at 30900 x g for 15 minutes at 4°C. The supernatant was taken to dryness and the residue was redissolved in 15 ml of the 2:1 (v/v) chloroform/methanol.

Microliter aliquots removed from this chloroform/methanol extract were used for determining fatty acid composition and the fatty acid to phosphorus ratio. Samples were prepared directly from this extract.

Preparation of Stearoyllysophosphatidylethanolamine

Stearoyllysophosphatidylethanolamine (SLPE) was prepared by a hydrogenation of the OLPE obtained from Serdary after the procedure by Klare Markley (77). An activated Raney nickel catalyst was prepared by slowly adding 2 g of inactive Raney nickel alloy (K & K Laboratories, Inc., Plainview, NY) to 50 ml of 20% (w/v) sodium hydroxide. The solution was

mildly boiled for 15 minutes and then was placed in a 30 ml teflon-lined screw cap test tube. After the catalyst settled, the base was poured off and the tube was filled with fresh 20% sodium hydroxide and capped. The mixture was then placed in an oven at 130°C for 2 hr, after which the base was suctioned off to a level which always ensured the catalyst remained covered and was never exposed to atmospheric oxygen.

Two liters of cold distilled water were used in approximately 29 ml washes to rid the catalyst of any sodium aluminate which would poison it. When the last visible trace of sodium aluminate precipitate was removed, the water was suctioned off and the catalyst was washed several times with absolute ethanol to remove residual water. The activated Raney nickel was then covered with 10 ml of absolute ethanol and stored in the capped test tube temporarily in a 130°C oven.

25 mg of OLPE were dried at room-temperature under a nitrogen stream. Based on the procedure of Van den Heuvel (78), 25 ml of absolute ethanol were added to the OLPE. This was then added along with a micro-size stir bar and half the activated Raney nickel catalyst to the hydrogenation apparatus (Parr Instrument Co., Moline, IL). The hydrogenation apparatus consisted of a 10 cm long by 3 cm diameter stainless steel cylinder topped with a 1000 psi USG gauge. A Polypenco Nylaflow tube connected the hydrogen source to a twist control flow knob on the gauge. The system was pressurized with 150 psi of hydrogen gas for 20 hr at room temperature with mild agitation.

The reaction mixture then was centrifuged at 30900 x g

for 15 minutes at 4°C in a Sorvall SS-34 head. The supernatant was poured off and the precipitate in each tube was rinsed with 30 ml of 2:1 chloroform/methanol and again centrifuged as before. The supernatants were combined and taken to dryness on a rotary evaporator. The SLPE was quantitatively transferred to a teflon-lined screw cap test tube, was taken to dryness, and the volume was adjusted to 5 ml with 2:1 chloroform/methanol.

Purification of Lysophosphatidylethanolamine with Sephadex LH-20

20 g of Sephadex LH-20 (the hydroxy propylated form of Sephadex G-25) were allowed to equilibrate in 200 ml of 2:1 chloroform/methanol at room temperature with gentle agitation overnight (the approximate bed volume of the LH-20 in chloroform/methanol is 4.05 ml per gram dry gel). The gel was then packed in a Pharmacia SR 25/45 organic solvent resistant column and rinsed with 300 ml of fresh 2:1 chloroform/methanol solvent.

Samples were siphoned onto the column and a 500 ml 2:1 chloroform/methanol reservoir was attached at the top of the column. The eluant flow rate was 2.5 ml per minute. 10 ml fractions were collected using a Technicon Time/Flow fraction collector. Forty fractions were collected and 0.1 ml aliquots were removed for a phosphorus determination. Tubes which resulted in the positive blue color of the Fiske-Subbarow (79) determination had their fractions analyzed by thin-layer with LPE standards to determine the type of phospholipid present. 0.1% ninhydrin in acetone was the developing reagent in this case.

Phosphorus determinations on SLPE fractions showed that fraction 6 yielded a phosphorus-containing compound. Thin-layer results on fraction 6 showed that with 0.1% ninhydrin in acetone as a color-developing reagent, one ethanolamine compound was present. Thin-layer using iodine vapors as color developer showed that the sample contained minute quantities of free fatty acids. It was this sample that was used for differential scanning calorimetry analyses.

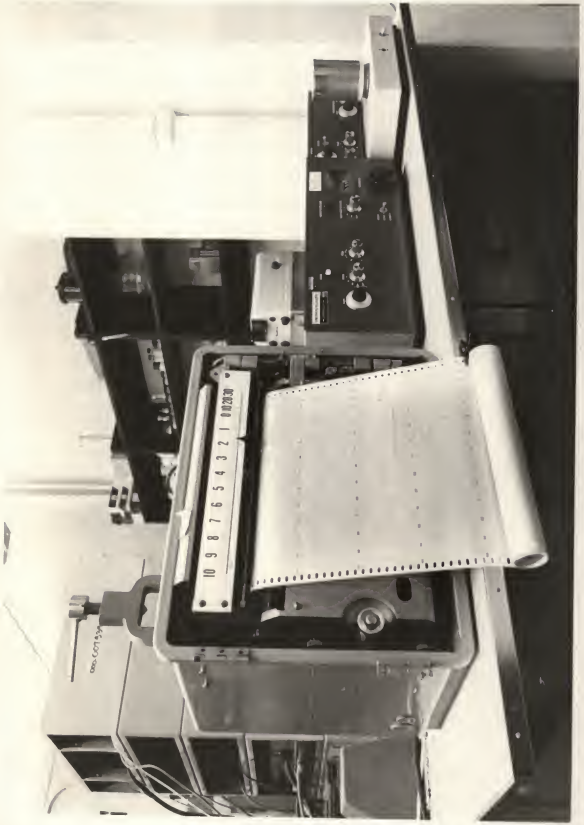
Preparation of Differential Scanning Calorimetry Samples

Approximately 4 mg aliquots of pure stock phospholipids were removed and placed in a small test tube. For cholesterol studies, the amounts of cholesterol needed for 0 to 60 mole percentages were computed (See Calculations) and the appropriate volumes of cholesterol stock were added. The samples were taken to dryness under a nitrogen stream and were placed in an evacuated dessicator overnight. 50 ul of 1:1 deionized water/ethylene glycol were added to each sample tube and lipid suspensions were made by heating and vigorously agitating the preparations on a Vortex mixer. Sufficient lipid was removed to fill a Perkin-Elmer aluminum sample pan (kit number 219-0062). Sample pan lids were placed on top of the pans and a vise was used to seal the sample pans.

Differential Scanning Calorimetry

The DSC samples were scanned on a Perkin-Elmer DSC-1B (See Figure 4). Sealed sample pans were placed in the sample compartment, the silvered Dewar liner was snapped in place, and the compartment was flushed with 10 psi of nitrogen gas for 5 minutes prior to a decrease in temperature. Upon addi-

Figure 4. The Perkin-Elmer DSC-1B Differential Scanning Calorimeter.


10 9 8 7 6 5 4 3 2 1

tion of liquid nitrogen to the Dewar liner, samples were allowed to cool to 60 degrees below their postulated transition temperatures before scanning. Samples were scanned at a rate of 10°C/minute. An appropriate range was chosen to keep the scan peak on scale and a slope was chosen to maintain the best stable baseline. Scans were recorded on a 5 millivolt Wheel-Co recorder (Barber-Colman) with a chart speed of 1 inch/minute.

Phosphorus Determination of Phospholipids from Calorimetry

After sufficient DSC scans were recorded, sample pans were sacrificed for phosphorus determinations (78). The sample pans were ruptured in a test tube to release the lipid and 1.0 ml of 2:1 chloroform/methanol was added. Samples were quickly vortexed to ensure that all LPE was dissolved. Three 0.1 ml aliquots were removed and the solvent was evaporated in a 130°C oven. Subsequently, 0.25 ml of 70% perchloric acid was added to each tube. Each tube was covered with a marble, inserted into a 220°C heat block, and allowed to digest overnight. The tubes were cooled and 4 ml of 2.5% (w/v) ammonium molybdate in 5 N sulfuric acid and 2 ml of 15% (w/v) sodium metabisulfite were added. A set of standards were prepared by taking 2, 4, 8, 10, 15, and 20 ug of phosphorus equivalent (monobasic potassium phosphate). The reagents were added to each tube and all tubes were mixed with a thin glass stirring rod. After proper mixing, all the tubes were placed in a wire basket and incubated in a boiling water bath. The incubation time in the boiling water bath was 45 minutes after which the tubes were removed from the bath.

After the samples were cooled, absorbances were measured at 830 nm on a Beckman DU-2 double beam spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Standard curves were prepared by least squares analyses of absorbance at 830 nm vs micrograms of phosphorus. Unknowns were calculated using the point-slope method.

DSC Scan Analysis

The areas under the transition peaks were calculated by using a Keuffel and Esser compensating polar planimeter after baselines were drawn. Baselines were drawn from the first point a transition deviated from a normal slope to the point a transition returned to a previous and/or a consistent new slope.

Preweighed samples of deionized water were run and the area of these scans were used to calibrate the instrument (See Calculations). This enabled computation of the heat of a thermotropic transition for a given compound.

Gas-Liquid Chromatography

The fatty acids of the lysophosphatidylethanolamines were analyzed for purity and the synthesized LPE's, i.e. the lauroyl and stearoyl derivatives, were analyzed qualitatively and quantitatively on a programmable Selecta-System/Series 5000 Barber-Colman gas chromatograph (Barber-Colman Co., Rockford, IL). The gas chromatograph column consisted of 7.5% diethyleneglycolsuccinate on Chromasorb G (80/100 mesh). Column temperature was maintained at 140^{o}C for the lauroyl and stearoyl qualitative determinations and a temperature programmed determination was used for LLPE quantitation with behenic (22:0)

methyl esters. Nitrogen was used as the carrier gas and was maintained at 15 lb pressure. The methyl esters were detected by a hydrogen flame ionization detector and peaks were recorded on a Honeywell recorder (Minneapolis-Honeywell Reg. Co., Brown Instrument Division, Philadelphia, PA).

Temperature programmed determinations had an initial isothermal period of 6 minutes at 140°C followed by a 3°C/minute increase in temperature to a final column temperature of 180°C.

Methyl esters of the LPE's were prepared by incubation of phospholipid sample with 1 ml of 10% boron trichloride in anhydrous methanol (w/v) at 60°C for 1 hr. After incubation, 1 ml of distilled water was added and the methyl esters were extracted three times with 1 ml volumes of hexanes. The hexane extracts were combined and washed with 0.5 ml distilled water. The water was drawn off and the hexanes were dried of residual water with anhydrous sodium sulfate. The resulting solvent was concentrated to approximately 50 ul and injected into the column.

Quantitation of fatty acids was accomplished with a known volume of LPE in 2:1 chloroform/methanol. Then, 0.1 ml of 5.044 mg/ml behenic acid in 2:1 chloroform/methanol was added and the solvents were evaporated. The esterification procedure proceeded as previously described.

Thin-Layer Chromatography

Thin-layer chromatography was done on commercial prepoured plates. Either 0.25 mm silica gel G Uniplates (Analtech, Inc., Newark, DE) or 0.25 mm silica gel 60 (without fluorescent indicator) 20 x 20 cm plates (EM Reagents, EM

Laboratories Inc., Elmsford, NY) were used. Standards that were used were DPPE, PLPE, and glycerophosphorylethanolamine. The solvent system was 65:25:4 chloroform/methanol/water exclusively. The color-developing reagents were iodine vapors (solid iodine in a developing tank) and 0.1% ninhydrin in acetone which was applied as a spray with nitrogen gas.

Plates were washed once in the 65:25:4 solvent and allowed to dry. This removed the iodine-developing contaminants from the plate in order to view free fatty acids at the solvent front. After sample application, the plates were developed in solvent.

CALCULATIONS:

Once the differential scanning calorimetry data had been collected, several calculations were necessary to obtain the thermal properties of the lysophosphatidylethanolamines. Each type of calculation performed is exemplified in this section.

Gas-Liquid Chromatography Peak Areas

The LPE fatty acyl compositions were analyzed by injecting methyl esters of the samples into a gas-liquid chromatograph. Ester peaks were detected and recorded on chart paper.

To calculate percentages of the various methyl esters, areas of the ester peaks were measured accordingly:

AREA = PEAK HEIGHT X PEAK WIDTH AT HALF HEIGHT

The areas were then summed and ester percentages were calculated as peak area multiplied by 100 the quantity divided by the sum of total peak areas.

Differential Scanning Calorimetry Peak Areas

DSC sample pans were prepared as described in Materials and Methods and scanning peaks which corresponded to the heats of phase transition were obtained. Peak areas were measured by the compensating polar planimeter, multiplied by the range setting (attenuation) of the instrument, and recorded. For pure LLPE, the average area for a particular peak was 219.625 which when multiplied by 2 for the range yielded a total area of 439.25 area units.

Differential Scanning Calorimetry Calibration Factor

Known weights of deionized water were scanned on the DSC and peak areas were measured and used to calibrate the heat per unit area. At $0^{o}C$, the Heat of Fusion of ice has been

cited as 79.7 cal/g (80). Using this information, a calibration factor of the instrument was obtained. When the Heat of Fusion in cal/g was multiplied by the weight of water in grams (this product represents the calories of heat of the ice to water phase transition) and when this product was divided by the product of the area of the peak multiplied by the range, the calibration factor in calories per unit area was obtained.

$$\text{CALIBRATION FACTOR} = \frac{79.7 \text{ cal/g water} \times \text{g water}}{\text{Area of ice to water peak} \times \text{Range}}$$

Heat of Transition

An average calibration factor was calculated from several deionized water scans and for the same particular sample, a factor of 3.559×10^{-5} cal/area unit was obtained. Thus, the heat of the gel to liquid-crystalline phase transition of a pure LLPE sample may be represented as the total area of the transition peak multiplied by the calibration factor, or:

$$439.25 \text{ area units} \times 3.559 \times 10^{-5} \text{cal/area unit} = 1.563 \times 10^{-2} \text{ cal}$$

Moles of LPE

Once the sample pans were scanned, the pans were then removed from the DSC and ruptured in a small test tube. The contents of the sample pans were then dissolved in 1 ml of chloroform/methanol (2:1) and 0.1 ml aliquots were analyzed for phosphorus as described in Materials and Methods. Moles of phospholipid present per sample pan were calculated in the following manner.

For the same particular LLPE sample, an average absorb-

ance at 830 nm of 0.702 was obtained. From a set of standards prepared at the same time, the ug of phosphorus were determined from a least squares analysis of the standards ($m = 9.923 \times 10^{-2}$, $b = 5.946 \times 10^{-2}$) and an average of 6.475 ug of phosphorus were present in the 0.1 ml aliquots of the particular LLPE sample pan.

Considering the dilution factors, the moles of phosphorus were calculated as follows:

$$1 \text{ ml} \times \frac{6.475 \text{ ug P}}{0.1 \text{ ml}} \times \frac{1 \text{ g P}}{1 \times 10^{6} \text{ ug P}} \times \frac{1 \text{ mole P}}{31 \text{ g P}} = 2.089 \times 10^{-6} \text{mole P}$$

Therefore, 2.089×10^{-6} moles P or 2.089 umoles of phosphorus were present in the sample pan of the particular LLPE sample.

From the structure depicted in Figure 3, there is only one atom of phosphorus present in one molecule of LPE. Acknowledging this relation, the umoles of phosphorus equal umoles of lysophospholipid and thus, 2.089 umoles of LLPE were present in the sample pan.

Enthalpy of Transition

To calculate the enthalpy of transition for the same particular LLPE sample, the calories of transition were converted to kilocalories and this value was then divided by the moles of LLPE to give the enthalpy of transition in Kcal/mole.

$$\frac{1.563 \times 10^{-2} \text{ cal} \times 1 \text{ Kcal}/1000 \text{ cal}}{2.089 \times 10^{-6} \text{ moles LLPE}} = 7.484 \text{ Kcal/mole}$$

Thus, the enthalpy of transition may be calculated in this manner. The enthalpy of transition for the particular pure LLPE has been calculated as 7.5 Kcal/mole.

Mole Percent Cholesterol

For cholesterol studies, various mole percent mixtures of lysophospholipid and cholesterol were studied. Mole percentages of cholesterol were computed with the following formula:

$$\text{MOLE \%} = \frac{\text{Y umole cholesterol}}{\text{Z umole LPE + Y umole cholesterol}} \times 100\%$$

The umoles of LPE were previously calculated by a phosphorus determination on the stock solution and a given amount of lysophospholipid was chosen to ensure that approximately 5 mg LPE were present in each mixing tube.

Once the umole of cholesterol were calculated for the desired mole % cholesterol with the specified LPE concentration, the necessary volumes of stock cholesterol (5.58 umoles/ml) were calculated. These exact volumes were rounded off to the most practical pipette value and the new exact mole % cholesterol was then recalculated.

For example:

$$\text{10 mole \%} = \frac{\text{Y umoles cholesterol}}{\text{12.04 umoles LPE + Y umoles cholesterol}} \times 100\%$$

After solving for Y:

$$Y = 1.438 \text{ umoles cholesterol}$$

Thus, the desired umoles of cholesterol for 10 mole % cholesterol (with 12.94 umole LLPE) are 1.438 umoles cholesterol. Solving for the exact necessary volume of stock cholesterol,

$$5.58 \text{ umole cholesterol/ml} \times W \text{ ml} = 1.438 \text{ umole cholesterol}$$

$$W \text{ ml} = 0.258 \text{ ml stock}$$

The practical volume to pipette is 0.25 ml of cholesterol stock. By reversing the above calculations, it is possible to determine the exact mole % cholesterol of a particular tube.

0.25 ml x 5.58 umole cholesterol/ml = 1.40 umole cholesterol

And:

$$V \text{ Mole } \% = \frac{1.40 \text{ umole cholesterol}}{12.94 \text{ umole LLPE} + 1.40 \text{ umole cholesterol}} \times 100\%$$

V Mole % = 9.7 mole % cholesterol

Enthalpies of transition for the various mole % cholesterol were calculated in the same manner as for the pure LLPE calculations.

Temperature of Transition

Temperatures of transition were calculated in the following manner. Normals were drawn to the scan baselines and the point where the peak first left the baseline was measured in mm to the nearest reference line on the chart paper. For all scans, reference points were 1 inch or 25.4 mm apart and corresponded to multiples of 10^{o} i.e. 400, 410, ... or 440, 450, ... These distances were divided by 25.4 mm and multiplied by 10 and then added to or subtracted from that reference point, depending if the point was above or below the reference.

For example, if a transition peak started at 8 mm above 410 instrument degrees, then this temperature was:

$$(8/25.4) \times 10 = 3.15$$

and:

$$3.15 + 410 = 413.15 \text{ instrument degrees}$$

Thus, the transition occurred at 413.15 instrument de-

grees.

Deionized water samples were used as calibration standards and the temperature where the transition started was taken as $0^{o}C$. To convert transition temperatures of LPE samples to ^{o}C, the average of the instrument temperatures for deionized water was subtracted from the average instrument temperature of the sample.

If the deionized water samples started their transition at 373.15, then the transition temperature of the sample in ^{o}C is:

$$413.15 - 373.15 = 40.0\ ^{o}C$$

RESULTS AND DISCUSSIONS:

The purity of each of tha LPE samples was determined by gas chromatographic analyses of the constituent fatty acids. The following results were obtained and represent the relative percentages which the desired methyl esters represent of the total esters observed.

L(12:0)LPE	87% lauric acid
P(16:0)LPE	99% palmitic acid
S(18:0)LPE	70% stearic acid
O($18:1^{\Delta 9}_{cis}$)LPE	68% oleic acid

All of the LPE samples when analyzed by thin-layer chromatography yielded one spot when developed with the ninhydrin developing reagent, verifying that only one ethanolamine compound was present. When duplicate plates were developed with iodine vapors, minute quantities of free fatty acids were detected with the LPE.

The lauroyl, palmitoyl, stearoyl, and oleoyl derivatives were analyzed by differential scanning calorimetry. No analyzable transition peaks were observed for the SLPE and OLPE derivatives. In the case of the SLPE, the transition temperature was determined from DSC scans, but no complete transition peaks were obtained. The OLPE was taken to the lowest temperature possible on the DSC-1B ($-90^{o}C$) and no thermal transition was observed between $-90^{o}C$ and $90^{o}C$. Differential scanning calorimetry was performed on LLPE and PLPE samples with and without added cholesterol to study the effects of cholesterol on the thermotropic transition of LPE. A typical series of scans for PLPE is shown in Figure 5.

Figure 5. A Series of Differential Scanning Calorimeter Scans of PLPE.

A - 0 mole % cholesterol

B - 10 mole % cholesterol

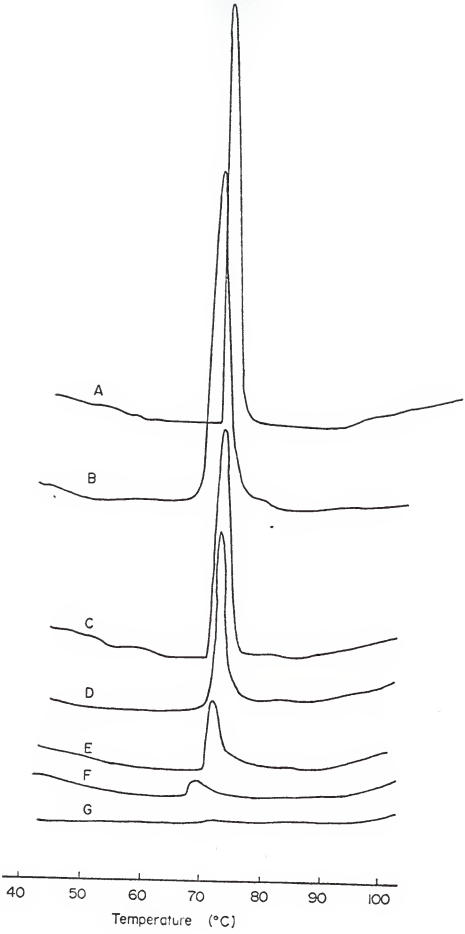
C - 20 mole % cholesterol

D - 30 mole % cholesterol

E - 40 mole % cholesterol

F - 50 mole % cholesterol

G - 60 mole % cholesterol


A
B
C
D
E
F
G
40
50
60
70
80
90
100
Temperature (°C)

Scan A represents the pure PLPE sample, or the 0 mole % cholesterol sample. A sharp endothermic phase transition is observed at 70.7°C for the pure compound, where the sharpness of the peak is a direct reflection of the high degree of cooperativity of the transition.

A cooperative transition on a DSC scan is denoted by a sharp peak, where many molecules undergo the phase transition at the same time. A less cooperative transition consists of a wider, more broad peak where the molecules undergo the transition more independently of one another (57).

Scan B of Figure 5 is the 10 mole % cholesterol scan. For LPE's, the addition of cholesterol decreases the cooperativity of the endothermic transition as indicated by the increased width of the peak. The insertion of cholesterol into the LPE micelles between the fatty acyl chains cause the hydrocarbon chains to isomerize more independently of each other (58) and instead of most of the fatty acyl chains undergoing the phase transitions at the same temperature, some "melt" at slightly lower temperatures and some at slightly higher temperatures, which causes an overall broadening of the DSC peak widths.

Discussions of cooperative units of a phase transition have appeared in the literature and there have been attempts to estimate the number of molecules in a cooperative unit for a given transition. A high cooperative unit number indicates an extremely cooperative phase transition and a low number indicates a less cooperative transition (55). The size of the cooperative unit has been related to the ratio of the width of

the phase transition to the calorimetric enthalpy (this is a simplification of the Van't Hoff equation; for further detail, see 83). However, the width of the phase transition varies with the method of detection. Different widths have been reported for the thermal transition of a compound when observed by fluorescence probes, differential scanning calorimetry, ^{13}CNMR, and ESR. At this time, there are too many variables in these determinations to enable many conclusions to be drawn from these data.

However, for both of the LPE's studied here (12:0 and 16:0), it can be concluded that cholesterol decreases the cooperativity of the gel to liquid-crystalline phase transition as it does for the other phospholipids studied (33-35,48,71).

The effects of increasing concentrations of cholesterol on the transition temperatures of both LLPE and PLPE were computed as described in Calculations. For a summary of the data, see Table II.

Upon analysis of the data with a one-way analysis of variance, the transition temperatures appear to be statistically different at the various mole percentages of cholesterol studied. The data were analyzed by least squares analyses and a slope of 0.011 was obtained for LLPE and a slope of -0.017 was obtained for the PLPE with correlation coefficients of 0.109 and -0.259 respectively. The low correlation coefficients obtained indicate that the slopes were not significant.

Thus, the effect of cholesterol on lysophosphatidylethanolamines produce no consistent response in the transition temperature, unlike data from lysophosphatidylcholines (71,81)

Table II. Transition Temperature and Mole Percent Cholesterol Data for LLPE and PLPE.

LLPE (12:0)		PLPE (16:0)	
Mole % Cholesterol	Transition Temperature	Mole % Cholesterol	Transition Temperature
0.0	40.9 43.4	0.0	70.2 70.7
8.2	44.3	11.8	68.9
10.0	41.4	22.3	70.3
11.1	43.3	32.9	71.4
20.0	42.9	43.3	71.1
21.9	40.6	53.4	67.7
25.5	41.2		
32.6	44.2		
40.1	43.1		

where the transition temperatures increase with increasing cholesterol concentrations. LPE interactions with cholesterol are also different than those of diacyl PC and PE with cholesterol where the transition temperatures decrease with increasing cholesterol (48,57).

When the transition temperatures of the pure LPE with different fatty acyl chains were plotted against carbon number of the hydrocarbon chains, a linear plot was obtained (See Figure 6). A summary of the data is found in Table III.

The transition temperatures for LLPE and PLPE were calculated as previously described and were taken from complete transition peaks. The SLPE derivative never gave a uniform peak on the DSC, but rather gave a reproducible "half-peak" at 81.8°C. The "half-peak" that was obtained for the SLPE derivative appeared as a normal peak, where the ascending limb of the peak was distinctly reproduced. However, once the top of the transition peak was reached, the pen of the recorder left the paper and never returned to the baseline, thus yielding the characteristic "half-peak".

When the SLPE point was plotted in Figure 6, this point reaffirmed the line obtained from the LLPE and PLPE derivatives within experimental error. Although we were unable to determine the transition temperature of the myristoyl derivative, we would expect it to occur at 56°C, based on the data plotted in Figure 6.

Thus, for the lysophosphatidylethanolamines studied here, there appears to be a linear relation between the temperature of transition and carbon number of the hydrocarbon chains.

Figure 6. A Plot of the Transition Temperature vs Hydrocarbon Chain Carbon Number.

□ - Expected Value

○ - Observed Value

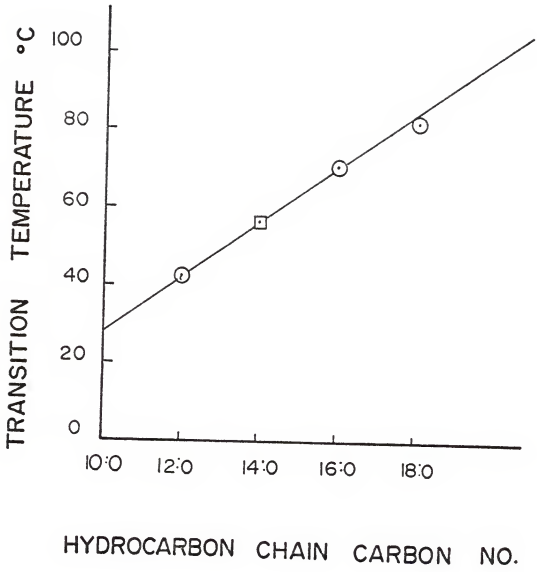

TRANSITION TEMPERATURE °C
100
80
60
40
20
0
10:0
12:0
14:0
16:0
18:0
HYDROCARBON CHAIN CARBON NO.

Table III. A Summary of LPE Calorimetric Data.

LPE	Transition Temperature ^{o}C (A)	(B)	ΔH_t (C)	Mole % Cholesterol (D)
12:0	41.74	42.33	7.2	50
16:0	70.47	70.43	8.5	60
18:0	81.80	-	-	-
$18:1^{\Delta 9}_{cis}$	-	-	-	-

A - Calculated as the average of the transition temperatures of the 0 mole % cholesterol samples minus the melting temperature of deionized water, which equals the temperature of transition in ^{o}C.

B - Values represent y-intercepts from the least squares analyses of transition temperature vs mole % cholesterol.

C - Represents the enthalpy of transition as Kcal/mole.

D - Mole % cholesterol required to eliminate the thermal transition as calculated by x-intercepts from least squares analyses of transition enthalpy vs mole % cholesterol.

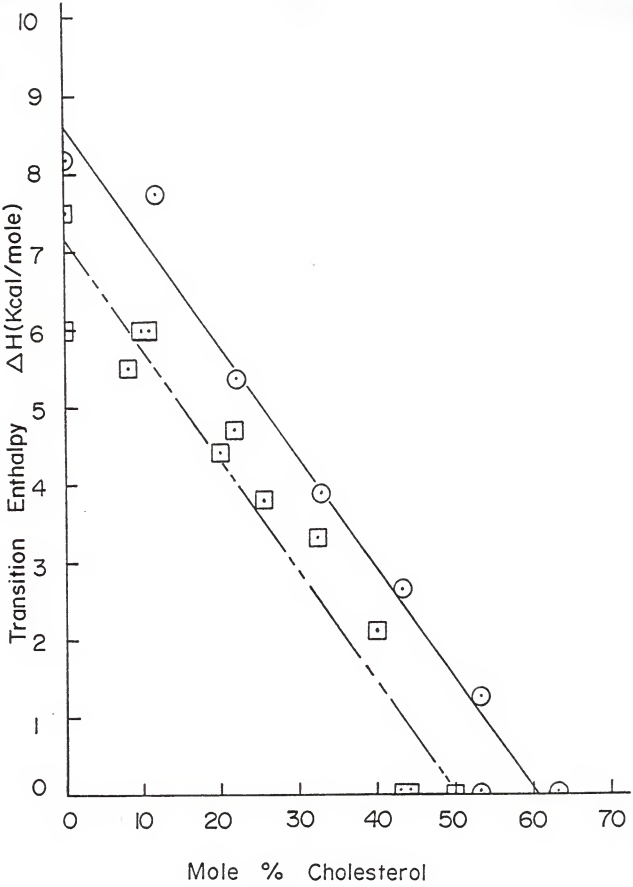
As described in the Calculations, several computations were required to obtain the enthalpies of transition. The resultant data are included in the Appendix.

An inverse linear relationship between the enthalpy of transition and the mole percent of cholesterol was obtained when transition enthalpy data were plotted against mole percentages of cholesterol (See Figure 7). As the mole percent of cholesterol increases, the transition enthalpy decreases for both LLPE and PLPE derivatives. The mole percentages of cholesterol necessary to eliminate the phase transitions are 50 and 60 mole % respectively.

The mole percentages of cholesterol necessary for obliteration of a thermotropic transition for diacyl phosphatidylcholines are 33 mole % (35,40) and 50 mole % (34,70) cholesterol. Hinz and Sturtevant (35) reported that a value of 33 mole % cholesterol was necessary to eliminate the transition peak of a PC DSC scan. In 1978, Mabrey, Mateo, and Sturtevant (70) reported that an error was made in citing the 33 mole % cholesterol value. Recent DSC experiments by Mabrey et al indicate that 50 mole % cholesterol eliminates the thermal transition of phosphatidylcholines. The reasons for the earlier value were that a calorimeter with a less stable baseline was used and sufficient amounts of phospholipids were not contained in the sample pans (70).

Less work has been done on diacyl phosphatidylethanolamines. Van Dijck et al (48) had reported that 33 mole % cholesterol eliminated the thermal transitions for both diacyl PC and PE. At this time, no values of 50 mole % cholesterol

Figure 7. A Plot of Transition Enthalpy vs Mole Percent Cholesterol of LLPE and PLPE.


Transition Enthalpy ΔH(Kcal/mole)
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
Mole % Cholesterol

have been reported for the elimination of a DSC transition peak for the diacyl PE compounds.

Klopfenstein (71,81) has reported that a value of 50 mole % cholesterol was necessary to eliminate the DSC phase transition for lysophosphatidylcholines. The LPC used in those studies included the myristoyl, palmitoyl, stearoyl, and oleoyl derivatives.

The mole percentages of cholesterol necessary for the elimination of the lauroyl and palmitoyl derivatives of LPE studied here are in accordance with the results of Klopfenstein's studies for LPC. However, it is not certain whether elimination of the DSC phase transition by 60 mole % cholesterol for PLPE is actually 50 mole % within experimental error or if the longer chain length LPE require higher concentrations of cholesterol to eliminate the phase transition. The former of the two explanations seems more plausible at this time.

The lines in Figure 7 showing the inverse relationship between enthalpy of transition and cholesterol concentration were obtained by least squares analyses. The y-intercepts, or the enthalpies of transition for pure LPE, are reported in Table III. For LLPE, the enthalpy of transition was calculated as 7.2 Kcal/mole as compared to 4.0 Kcal/mole for DLPE (48). For PLPE, the transition enthalpy was calculated as 8.5 Kcal/mole which was the same value reported for DPPE (48). From the data comparisons, it is clear that the removal of one fatty acid chain does not reduce the transition enthalpy. In the case of LLPE, the transition enthalpy was greater than

the corresponding diacyl PE and in the case of PLPE, the transition enthalpy was equal to the DPPE.

The removal of the hydrocarbon chain from the C_2 position of the glycerol skeleton does not exert the same effects for the two different polar headgroups. By comparing the results of the LPE from Table III with the results from Table I, several conclusions can be drawn.

The average difference in transition temperature of PC and LPC having tha same acyl chains is approximately $40^{o}C$ where the LPC derivative has a lower transition temperature than the PC. Conversely, the average difference in transition temperatures of PE and LPE having identical hydrocarbon chains is approximately $12^{o}C$ where the transition temperature of the LPE derivatives are higher than the PE derivatives. No reason for this difference is apparent at this time.

A comparison of the transition enthalpies of the diacyl PC with the corresponding lyso derivative reveals that loss of the fatty acid results in a decrease in the transition enthalpy of approximately 2 Kcal/mole. However, comparison of the enthalpy of transition for the palmitoyl derivatives of PC shows the lyso compound to have the same enthalpy of transition as the diacyl compound. With the lauroyl derivatives of PE, the lyso derivative had a 3 Kcal/mole higher enthalpy of transition than the diacyl compound. These results suggest that whereas the LPC are suspended in aqueous systems as micelles, the LPE exist in a structure in which the molecules have greater opportunity for inter-molecular interaction, thus forming a more stable structure which requires more heat to

disrupt.

Introduction of cis unsaturations apparently has a dramatic effect on the transition temperature of lysophospholipids. In this study, a transition for the OLPE was not observed down to -90°C which is in agreement with the results of Klopfenstein (71) where no transition was observed for the corresponding LPC down to -80°C.

The SLPE did not yield true thermotropic transition peaks on the DSC scans even though the compound was chromatographically pure. When the 50 ul of 50%-50% ethylene glycol/deionized water were added to the SLPE derivative, followed by mixing and heating, the lysolipid suspension became as clear as the other LPE's, indicating a liquid-crystalline phase formation. However, the SLPE appeared to have a solubility problem in the ethylene glycol/water solvent. The SLPE precipitated out of solution as the sample cooled down, making it difficult to transfer sufficient amounts of lipid into the calorimeter cells. No explanation is apparent as to why this compound did not exhibit transition behavior similar to that observed with the LLPE and PLPE compounds.

SUMMARY:

The thermal properties of lysophosphatidylethanolamine derivatives were studied by differential scanning calorimetry. Lauroyl, palmitoyl, and stearoyl derivatives were observed to undergo gel to liquid-crystalline phase transitions at 41.7°C, 70.5°C, and 81.8°C respectively. No transition was observed for the oleoyl derivative within the detection limits of the instrument. Transition enthalpies of 7.2 Kcal/mole and 8.5 Kcal/mole were observed for LLPE and PLPE respectively. It was not possible to calculate a transition enthalpy for the stearoyl derivative due to the unusual DSC behavior it exhibited.

Comparison of the observed transition temperature and transition enthalpy for LLPE with literature values for DLPE showed that the transition temperature was 12°C higher and the transition enthalpy was twice as great for the lyso derivative, suggesting that the lysocompound formed a more stable structure. When the same comparisons were made for the palmitoyl compounds, the transition temperatures were 12°C higher, but the transition enthalpies were equal. The explanation for these data is unclear.

The variation of transition temperatures of LPE with chain length indicated that increased hydrocarbon chain length increased the LPE transition temperature, which agrees with the results of Chapman et al (33). Thus, the LPE's as a group fit into the pattern observed with numerous other classes of lipids in that the fatty acids esterified have a large role in determining the physical properties of the lipid

i.e. the fluidity of a synthetic membrane.

Since a lysophosphatidylethanolamine has only one hydrocarbon chain and diacyl phosphatidylethanolamines have two chains, the enthalpy of transition of the LPE might be expected to be only half that of the PE if their hydrocarbon chains were subjected to the same association forces. The results of this study clearly do not substantiate that notion. Therefore, the hydrocarbon chains of LPE derivatives must be more tightly associated.

Cholesterol interactions with LPE were studied also. A negative linear correlation was observed between enthalpy of transition and the mole percent of cholesterol present in the LLPE and PLPE systems. Values of at least 50 mole % cholesterol were necessary to eliminate the thermal transitions of LLPE and PLPE.

The addition of cholesterol to the LLPE and PLPE systems caused a broadening of the DSC peaks. This suggested that cholesterol decreased the cooperativity of the LPE systems, allowing the lipid clusters to undergo the gel to liquid-crystalline phase transition more independently of one another.

These results appear to support Ladbrooke's theory (36) concerning the role of cholesterol in membranes. Ladbrooke proposed that cholesterol in a membrane expands the temperature range over which the membrane phospholipids maintain a fluid state, although the fatty acids of the lipid would still determine the temperature at which the thermal transition takes place.

Results of this study clearly indicate that the enthalpy of transition of a diacyl PE should not be divided equally per hydrocarbon chain as one hydrocarbon chain had greater or equal transition enthalpies. Also, results presented here support the data of experiments on other phospholipids which have indicated that the fatty acyl chain lengths of the phospholipids dictate the temperature of membrane fluidity and cholesterol is instrumental in extending the temperature range of fluidity.

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APPENDIX:

LLPE Calibration Factor

The following data were obtained for a 0.00144 g sample of deionized water.

Average Transition Temperature: 373.84 ----- $0^{o}C$

Calibration Factor = $\frac{79.7 \text{ cal/g water} \times 0.00144 \text{ g water}}{2968 \text{ area units}}$

$= 3.867 \times 10^{-5}$ cal/unit area

LLPE Calorimetric Data:

The above calibration factor was used to calculate information for the following LLPE samples:

SAMPLE (Mole % Cholesterol)	CALORIES OF TRANSITION ($\times 10^{2}$)	MOLES OF PHOSPHOLIPID ($\times 10^{6}$)	ENTHALPY OF TRANSITION (Kcal/mole)	TRANSITION TEMPERATURE (^{o}C)
0.0	1.491	2.483	6.0	40.89
8.2	2.232	4.064	5.5	44.89
10.0	1.527	2.564	- 6.0	41.43
20.0	1.384	3.183	4.4	42.85
25.5	1.287	2.243	3.8	41.18
40.1	0.604	2.933	2.1	43.05
44.4	0.0	2.023	0.0	-
50.0	0.0	2.365	0.0	-

A second set of data for the LLPE derivative was observed using the following deionized water data.

Average Transition Temperature: 374.23 ----- $0^{o}C$

Calibration Factor = 3.559×10^{-5} cal/unit area

SAMPLE (Mole % Cholesterol)	CALORIES OF TRANSITION ($\times 10^2$)	MOLES OF PHOSPHOLIPID ($\times 10^6$)	ENTHALPY OF TRANSITION (Kcal/mole)	TRANSITION TEMPERATURE (^oC)
0.0	1.563	2.089	7.5	43.45
11.1	1.419	2.381	6.0	43.32
21.9	1.612	2.474	4.7	40.59
32.6	0.562	1.704	3.3	44.19
42.9	0.0	4.088	0.0	-

LLPE Statistical Results

A plot of the transition enthalpy vs mole % cholesterol was made from the two sets of data and the line equation which resulted from a least squares analysis was:

$$y = (-0.1435)\,x + 7.17$$

x-intercept = 50.02 mole % cholesterol

y-intercept = 7.2 Kcal/mole

$$r = -0.9657$$

For 13 samples, the degrees of freedom are equal to 12, and at an alpha level of 0.05, with H_o: $\rho = 0$ vs H_a: $\rho \neq 0$, the critical value is -0.532. Since the $r_{calculated}$ is more negative than -0.532, then H_o is rejected and there is a correlation of the transition enthalpy vs mole % cholesterol.

A one-way analysis of variance was performed on the transition temperatures of the LLPE at various mole percentages of cholesterol. The results of the analysis indicate that at the alpha level of 0.05, the F statistic shows significant difference among the transition temperatures at the corresponding mole percentages of cholesterol. Thus, at the 95% level, not all the transition temperatures are experimentally

equal.

A least squares analysis was performed on the data to determine whether the increasing concentration of cholesterol had a positive or negative effect on the transition temperature.

^{o}C Transition Temperature (Y)	Mole % Cholesterol (X)
40.89	0.0
43.45	0.0
44.29	8.2
41.43	10.0
43.28	11.1
42.85	20.0
40.59	21.9
41.18	25.5
44.19	32.6
43.05	40.1

The line equation resulting from the least squares analysis was:

$$y = (0.0115)\, x + 42.33$$

Thus, the transition temperature in ^{o}C of pure LLPE is 42.33^{o}C by least squares analysis with a correlation coefficient of r = 0.1085.

Since there were 10 samples, there were 9 degrees of freedom. At the alpha level of 0.05 and by choosing the null and alternate hypotheses as $H_o: \rho = 0$ vs $H_a: \rho \neq 0$, a test was designed to check the significance of the correlation coefficient. From a table of correlation coefficients at the

5% level of significance (84), the critical value was determined as 0.602. Thus, when $r_{calculated}$ was greater than 0.602, the r was significantly different from zero. In this case, $r_{calculated}$ was less than 0.602. Therefore, the null hypothesis was accepted and there was no apparent correlation of the transition temperature and the mole % cholesterol.

PLPE Calibration Factor

For 0.00025 g deionized water, the following data was obtained.

Average Transition Temperature: 372.56 ----- $0^{o}C$

$$\text{Calibration Factor} = \frac{79.7 \text{ cal/g water} \times 0.00025 \text{ g water}}{567 \text{ area units}}$$

$$= 3.514 \times 10^{-5} \text{ cal/unit area}$$

PLPE Calorimetric Data

The above calibration factor was used to calculate the following PLPE information:

SAMPLE (Mole % Cholesterol)	CALORIES OF TRANSITION ($\times 10^2$)	MOLES OF PHOSPHOLIPID ($\times 10^6$)	ENTHALPY OF TRANSITION (Kcal/mole)	TRANSITION TEMPERATURE (^{o}C)
0.0	1.148	1.495	7.7	70.22
0.0	0.917	1.056	8.7	70.68
11.8	0.686	0.890	7.7	68.85
22.3	0.685	1.231	5.3	70.33
32.9	0.406	1.067	3.8	71.44
43.3	0.410	1.550	2.6	71.08
53.4	0.197	1.550	1.3	67.65
53.4	0.0	1.517	0.0	-
63.4	0.0	0.879	0.0	-

PLPE Statistical Results

A plot of the enthalpy of transition as Kcal/mole vs mole % cholesterol was made and a least squares analysis was performed on the data. Assigning mole % cholesterol as x values and the transition enthalpy as y values, the results obtained were as follows:

$$y = (-0.140)\,x + 8.49$$

x-intercept = 60.60 mole % cholesterol

y-intercept = 8.5 Kcal/mole

$$r = -0.9853$$

where the transition enthalpy for pure PLPE is 8.5 Kcal/mole and 60 mole % cholesterol eliminates the gel to liquid-crystalline transition.

A one-way analysis of variance was performed on the PLPE data. At the 0.05 alpha level, the F statistic was in the critical region and all of the transition temperatures at the corresponding mole percentages of cholesterol were not experimentally equal. Thus, at the 95% level of significance, the transition temperatures differed significantly at varied mole percentages of cholesterol.

In order to determine that the increasing mole percentages of cholesterol had either a positive or negative effect on the transition temperature, a least squares analysis was performed on the data.

^{o}C Transition Temperature (Y)	Mole % Cholesterol (X)
70.22	0.0
70.68	0.0

^{o}C Transition Temperature (Y)	Mole % Cholesterol (X)
68.85	11.8
70.33	22.3
71.44	32.9
71.08	43.3
67.65	53.4

The line equation resulting from the least squares analysis was:

$$y = (-0.0166)\ x\ +\ 70.43$$

Thus, the transition temperature in ^{o}C of pure PLPE was determined at $70.43^{o}C$ by least squares analysis with a correlation coefficient of $r = -0.2594$.

For the samples, there were 6 degrees of freedom and at an alpha level of 0.05 with $H_o: \rho = 0$ vs $H_a: \rho \neq 0$, the critical region according to Zar (84) is -0.707. Since $r_{calculated}$ is not more negative than -0.707, the correlation coefficient of the data is not significantly greater than zero, so there is no correlation of the transition temperature and the mole % cholesterol data.

THERMAL PROPERTIES OF LYSOPHOSPHATIDYLETHANOLAMINES

by

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The thermal properties of lysophosphatidylethanolamines (LPE) and LPE interactions with cholesterol were studied by differential scanning calorimetry (DSC). Methods were developed for synthesis of the lauroyl and stearoyl derivatives and thermal properties were reported for the lauroyl, palmitoyl, stearoyl, and oleoyl derivatives of LPE.

Enthalpies for the gel to liquid-crystalline phase transition of lauroyllysophosphatidylethanolamine (LLPE) and palmitoylphosphatidylethanolamine (PLPE) were calculated and values of 7.2 Kcal/mole and 8.5 Kcal/mole respectively were reported. The stearoyl derivative (SLPE) exhibited unusual DSC behavior and no transition enthalpy was obtained. Also, no phase transition was detected for oleoyllysophosphatidylethanolamine (OLPE) from -90°C to 90°C.

Transition enthalpies for LLPE and PLPE derivatives were compared to the enthalpies of the corresponding diacyl phosphatidylethanolamines (PE). The transition enthalpy of the LLPE was approximately twice as great as that reported for the dilauroylPE (DLPE). The transition enthalpy of the PLPE was the same as that reported for dipalmitoylPE (DPPE). Thus, the effect of the absence of a hydrocarbon chain from a PE upon the transition enthalpy indicated that when referring to a diacyl PE, each chain should not be ascribed half of the transition enthalpy.

The gel to liquid-crystal phase transition temperatures were measured by DSC for LLPE, PLPE, and SLPE derivatives and values of 41.7°C, 70.5°C, and 81.8°C respectively were obtained. LPE hydrocarbon chain carbon number and transition tem-

perature appeared to have a positive linear correlation. A transition temperature for the myristoyl derivative (MLPE) was estimated at 56°C from a plot of transition temperature vs LPE hydrocarbon chain carbon number.

Transition temperature differences between diacyl PE and LPE were compared to transition temperature differences of diacyl phosphatidylcholines (PC) and lysophosphatidylcholines (LPC). The LPC transition temperatures were approximately 40°C below the corresponding diacyl PC whereas the LPE transition temperatures were approximately 12°C above the corresponding diacyl PE.

The effects of cholesterol on the thermal properties of LPE were studied also. Increasing concentrations of cholesterol decreased the enthalpy of transition of the LLPE and PLPE derivatives. The transition enthalpy vs mole percent cholesterol exhibited a negative linear correlation. Values of at least 50 mole percent cholesterol were necessary to eliminate the thermal transitions of LLPE and PLPE. No cholesterol studies were reported for the SLPE and OLPE as complete DSC scans were not obtained for these derivatives.

Increasing concentrations of cholesterol had no significantly consistent effects of the transition temperatures of LLPE and PLPE, unlike the cholesterol studies of other phospholipids. LPC interactions with increasing cholesterol concentrations increased the transition temperature of the lysolipid system. When compared to the diacyl phospholipid systems, PC and PE interactions with increasing cholesterol decreased the transition temperatures.