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Characterization of cholestrylhemisuccinate- and tocopherol acid succinate- phospholipid membranes

Author
Lai, Ming-Zong,

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CHARACTERIZATION OF CHOLESTERYLHEMISUCCINATE- AND TOCOPHEROL ACID SUCCINATE - PHOSPHOLIPID MEMBRANES: PHASE BEHAVIOR OF pH-SENSITIVE LIPOSOMES

by

MING-ZONG LAI

B.S., NATIONAL TAIWAN UNIVERSITY, 1974

M.S., UNIVERSITY OF ILLINOIS, 1979

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To My Father and Mother

Acknowledgement

I wish to express my sincere thanks to Dr. Frank Szoka, my advisor, for his guidance, support and encouragement throughout the course of my study. I have particularly benefited from his scientific perceptions.

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Finally, I have to extend my appreciation to the City and Bay of San Francisco - for reasons needless to say.

Ming-Zong Lai

June 1984

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List of Abbreviations

CHEMS	Cholesterylhemisuccinate
DMPE	Dimyristoylphosphatidylethanolamine
DPPC	Dipalmitoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DSC	Differential Scanning Calorimetry
EM	Electron Microscopy
EPC	Egg Phosphatidylcholine
H_{II} Phase	Hexagonal Phase
L-H	Lamellar to Hexagonal
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PE	Phsophatidylethanolamine
PG	Phosphatidylglycerol
^{31}P-NMR	Phosphorous-31 Nuclear Magnetic Resonance
TS	Tocopherol Acid Succinate
TPE	Phosphatidylethanolamine Prepared From Egg Phosphatidylcholine by Transesterification
Tris	Tris-(hydroxylmethyl)aminomethane Hydrochloride

Abstract

The effectiveness of liposomal carriers to selectively release drugs can be enhanced by using lipid compositions that exploit the local environments of tissues. We have used cholesterylhemisuccinate (CHEMS) and tocopherol acid succinate (TS) to prepare liposomes that are sensitive to changes in pH. In order to understand the molecular mechanisms which control these liposomes, the pH-sensitive phase behavior and the interactions of CHEMS/TS with phospholipids were examined.

At neutral pH, both CHEMS and TS can dissolve in phospholipid bilayers to an extent greater than their parent compounds, cholesterol and tocopherol, respectively. The charged form of CHEMS and TS also stabilize phosphatidylethanolamine (PE), a non-bilayer forming lipid, in a bilayer phase. The thermotropic behavior of CHEMS-phospholipid mixtures, as studied by differential scanning calorimetry, is identical to that of cholesterol-phospholipid despite the removal of the 3β-OH from CHEMS. This indicates that 3β-OH group is not necessary for the formation of a sterol-phospholipid complex. In the case of tocopherol, the substitution of the hydroxyl group with a succinate group significantly reduces its interaction with phospholipids as measured by differential scanning calorimetry.

Neutralization of CHEMS in CHEMS-PE mixtures by protons or calcium ions immediately aggregates the CHEMS-PE liposomes. At low pH, CHEMS remains effectively complexed with the phospholipid, and macroscopic phase separation does not occur. The association of protonated CHEMS with PE promotes the formation of the hexagonal phase. The chelation of CHEMS by calcium ions, however, stabilizes PE in the lamellar phase.

Protons also promote the formation of non-bilayer structures in TS-phospholipid membranes. In the case of DPPC with 60 mole% TS at pH 4.5, a ^{31}P-NMR spectrum characteristic of a hexagonal phase is generated, while X-ray diffraction and freeze fracture electron microscopy clearly demonstrated a mixture of bilayer and planar lamellar structures.

These results have demonstrated that the incorporation of CHEMS or TS into phospholipid bilayers confers an acid-sensitivity on the liposomes. Although the interaction with phospholipids is different for CHEMS and TS, both succinate esters stabilize the phosphatidylethanolamine in bilayer membranes at neutral pH. However the protonation of CHEMS and TS that occurs at low pH promotes the formation of non-bilayer structures from such mixtures.

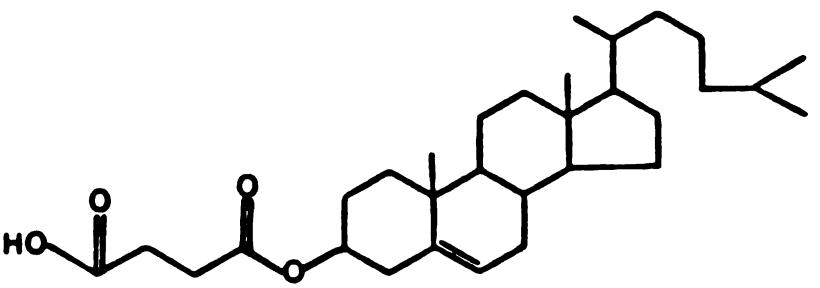
CHAPTER I

INTRODUCTION

1. Objectives

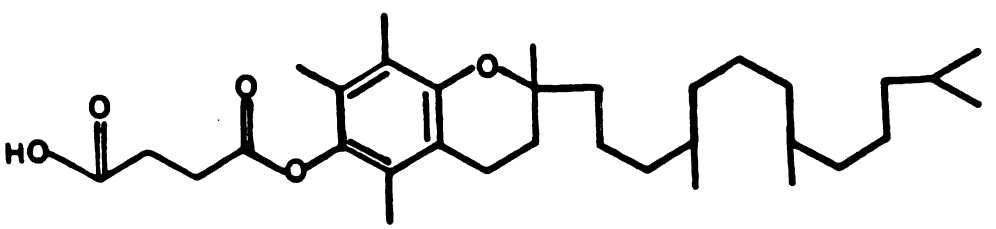
The use of lipid vesicles as drug carriers has promoted the search for lipid compositions that can exploit local environments found in the body to selectively release drugs. Both temperature and localized pH gradients have been explored as a mean to bring about localized release of liposome encapsulated drugs. Recently lipid compositions have been proposed that can destabilize the bilayer as the pH is lowered from 7 to 5 resulting in the release of entrapped compounds from the liposome. Although it has been hypothesized that the release results from a transition of the bilayer structure to a hexagonal H_{II} phase, the exact molecular changes that occur have not yet been characterized.

The purpose of this thesis is to characterize the interaction of two acid-sensitive derivatives, CHEMS and TS(Fig. I-1), with phospholipids; and to elucidate the membrane structural changes that accompany the pH titration of the carboxylate group when these compounds are incorporated into membranes. Since both compounds are derivatized through the hydroxyl group they can also serve as model com-



CHOLESTERYL HEMISUCCINATE

(CHEMS)



TOCOPHEROL ACID SUCCINATE

(TS)

Figure I-1. Structure of cholesterylhemisuccinate(CHEMS) and tocopherol acid succinate(TS).

pounds to delineate the role of the hydroxy group in their interaction with phospholipids. This is a particularly interesting question since the β-hydroxyl group of cholesterol has been considered to be necessary for cholesterol's interaction with phospholipids(Huang, 1977; Presti et al., 1982).

2. Rationale

2.1 Liposomes are taken up by endocytosis

Liposomes have been widely used as carriers to deliver drugs and macromolecules to cells and tissues(for review see Gregoriadis, 1976; Kimbelberg and Mayhew, 1978; Papahadjopoulos et al., 1981). As carriers, liposomes exhibit a relative low toxicity, have the capacity of carrying both hydrophilic and hydrophobic molecules, protect encapsulated compounds from exogenous enzymatic degradation, and can be modified on the surface with ligands that mediate site specific delivery. However, the delivery of molecules into cells is dependent on the mechanism by which liposomes enter the cells. Figure I-2 shows that liposome-cell interactions may occur in four different manners: lipid exchange, stable adsorption, fusion and endocytosis. Only through fusion and endocytosis may liposomes deliver their content into cells. Studies have shown that liposomes enter cells mainly through the endocytosis route(Szoka et al., 1979, 1980; Struck et al., 1981; Straubinger et al., 1983). Thus, most of the

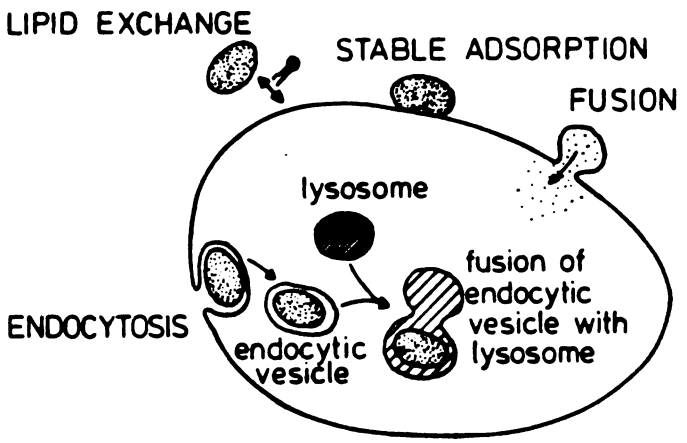


Figure I-2. Major mechanisms of liposome-cell interactions. (Reproduced with permission of J. Dijkstra)

liposome-associated molecules are delivered into lysosomes. This is correlated with the relatively high efficacy of liposome-encapsulated agents in the treatment of lysosome associated disorders such as lesishmania(New et al., 1978; Alving et al., 1978) and metal poisoning(Rahman, 1980). Compounds of high molecular weight, charged molecules or those labile to lysosomal enzymes would most likely be trapped and/or degraded in the lysosome. Therefore, for such compounds, liposomes would not be particularly effective for enhancing their delivery into the cytosol.

2.2 Endocytosis and associated low pH compartments

Endocytosis is the cellular process by which exogenous molecules can be internalized into the cell via plasma membrane-derived vesicles and vacuoles. The details of how microparticulates are internalized is dependent on the size of the particle and whether or not it can interact with a specific receptor on the cell surface. A variety of biologically active macromolecules such as insulin, transferrin, low-density lipoproteins and asialoglycoproteins are bound to cell-surface receptors and internalized in structures known as coated vesicles(Brown et al., 1979; Pastan and Willingham, 1981; Fig. I-3, [2]). The coated vesicles then shed their "coat", which is principally composed of a protein complex called clathrin, and fuse with one another to form endosomes. Small molecules that do not bind to surface receptors can be taken up through a process called fluid-

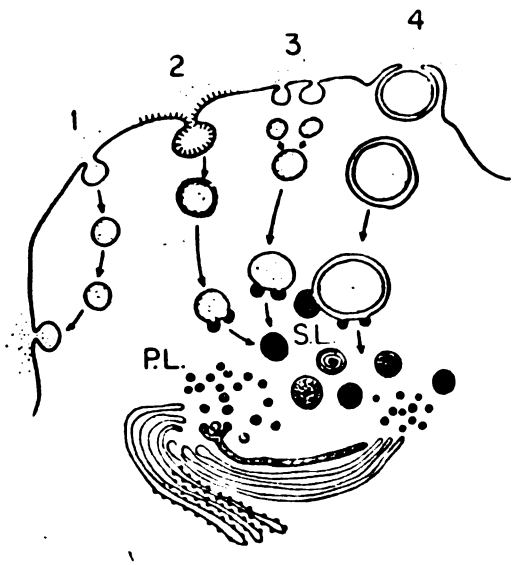


Figure I-3. Schematic representation of various endocytic pathways. (1) Fluid-phase pinocytosis: the vesicles transverse cytoplasm without fusing with lysosomes. (2) Receptor mediated endocytosis(adsorptive endocytosis): the endocytic vesicle then fuses with primary (P.L.) or secondary lysosomes (S.L.). (3) Fluid-phase pinocytosis: pinocytic vesicles fuse with each other and subsequently fuse with lysosomes. (4) Phagocytosis: the phagosome encloses the particle and usually fuses with primary or secondary lysosomes to form phagolysosomes. (Silverstein et al., 1977; Reproduced with permission, from the Annual Review of Biochemistry, vol. 46 (c) 1977 by Annual Reviews Inc.)

phase endocytosis in a structure called a pinosome(Pastan and Willingham, 1981; Fig. I-3, [1,3]). Particulate substances may enter phagocytic cells by a nonspecific uptake process in a vacuole termed a phagosome(Silverstein et al., 1977; Fig. I-2, [4]). Endosomes, phagosomes and some pinosomes then fuse with lysosomes to form structures known as secondary lysosomes(Silverstein et al., 1977).

Based on the acid-pH optimum of the lysosomal hydrolase, the pH inside the lysosome has been proposed to be relatively acidic(de Duve and Wattiaux, 1966), a phenomenon that was described in the early observations of Metchnikoff(1893). The direct measurement of intralysosomal pH in living cells using pH-sensitive fluorescent probes yields a pH value of 4.7-4.8(Ohkuma and Poole, 1978). The acidification of phagosomes in macrophages has been shown to begin within 5 min of phagosome formation, prior to fusion with lysosomes(Geisow et al., 1981). Recently, endosomes containing α_2-macroglobulin (Tycko and Maxfield, 1982), transferrin (van Renswoude et al., 1982) and asialoglycoprotein (Tycko et al., 1983) have been shown to have a pH in the range of 5.0-5.8. The acidification process has also been demonstrated to occur in the coated vesicles isolated from bovine brains, and is correlated with the presence of a proton-translocating ATPase on the coated vesicles(Forgac et al., 1983; Xie et al., 1983).

Acidification of the endosomal compartment is believed to be an important part of the mechanism of receptor recycling (Brown et al., 1983; Harford et al., 1983). Many ligands dissociate from their receptors at pH < 6. This permits the sorting of the unoccupied receptors that can then return to the cell surface, while keeping the ligand inside the cell. Moreover, the low pH environment of the endosome is utilized by a variety of viruses including influenza virus, Semliki Forest virus and vesicular stomatitis virus to gain access to the cytoplasm of the host cells. These viruses bind to cell surface receptors and enter endosomes where the acidic pH triggers the fusion of the viral envelope with the endosome membrane, resulting in the transfer of the nucleocapsids into the cytoplasm(Helenius et al., 1980; Maltin et al., 1981, 1982; Marsh et al., 1983a). This membrane fusion is mediated by a viral glycoprotein that undergoes a conformational change at low pH(Skehel et al., 1982). The reconstitution of Semliki Forest virus spike proteins into lipid bilayers produces liposomes that fuse with the plasma membrane at low pH's(Marsh et al., 1983b).

In addition, a number of "opportunistic" ligands use the acidic pH to gain access to the cytosol. Recent studies suggest that subsequent to the uptake of diphtheria and tetanus toxins by cells through endocytosis, the toxins form transmembrane channels in the endosomal membrane and enter

the cytoplasm(Donovan et al., 1981; Boquet and Duflet, 1982). Thus the endosomal compartment appears to be rapidly acidified and this change in pH can be exploited by biological and viral molecules to gain access to the cytoplasm of cells.

2.3 pH-sensitive Liposomes

The observation that liposomes are internalized via the endocytic pathway suggests that the delivery of liposome-encapsulated contents into the cytosol might be improved if the liposome can be designed to utilize the acidity of the endocytic compartment to enhance penetration of encapsulated compounds into the cytosol. Either a fast release of entrapped compounds in the endosome to create a concentration gradient across the membrane, or an acid-induced fusion between the liposomal membrane and endocytic vesicle-membrane may enhance the cytosol delivery. Although acid-sensitive liposome can be prepared by the incorporation of viral fusion proteins into lipid bilayers(Marsh et al., 1983b), there are difficulties inherent with the reconstituted virosomes. The isolation of viral protein is laborious even with proteins that can be produced by cloning methods(Porter et al., 1979). Additional concerns on the stability, ease of preparation, cost and toxicity suggest that virosome will not be an ideal carrier. This encouraged us to devise liposome compositions that could undergo a pH dependent conformational change without the use of viral

proteins.

2.4 Design of pH-sensitive Liposomes

The property of phosphatidylethanolamine to undergo a transition from a bilayer to a hexagonal phase(see Fig. I-8, Sec. 3.2.) at physiological conditions can be used to produce the required membrane destabilization at low pH. It is clear that the integrity of a bilayer membrane will not be maintained in the presence of a hexagonal structure, and should result in the release of liposome contents(Cullis and de Kruijff, 1979; Siegel, 1984). In addition hexagonal structures have also been implicated in membrane fusion(Cullis and Hope, 1978; Cullis and de Kruijff, 1979), although this is not a universally accepted finding(Bearer et al., 1982; Ellens et al., unpublished result).

As will be discussed in section 3.2., the inability of phosphatidylethanolamine to adopt the bilayer structure is attributed to specific head group interactions. PE can adopt the bilayer structure if this specific interaction is disrupted, for example, by chemical modification of the primary amine on the head-group or by the incorporation of other amphiphiles. One way of introducing acid-sensitivity to the PE system is to chemically modify the ethanolamine head-group with an acid-sensitive derivative. This would disrupt the normal PE head-group interaction and permit the bilayer to form. However as the pH is lowered the derivative would be hydrolyzed and unmodified PE would then

rearrange into a hexagonal structure. An alternative way to bring about a pH induced destabilization is by including an amphiphile which is sensitive to pH < 6. Protonation of the amphiphile should result in an increase in its hydrophobicity and alter its distribution or head-group area in the bilayer. This would permit the PE to undergo a transition into the hexagonal phase.

A number of ligands used in the reversible blocking of amino groups in proteins can be conjugated to PE. However, the rate of hydrolysis of the PE derivatives at low pH is critical for the success of the system. Modification of PE with ligands such as the maleyl group is impractical, because the half life for hydrolysis of maleyl-lysine is 11 hr at pH 3.5(Butler et al., 1969). Given the rapid rate of internalization and degradation of liposomes by cells (Dijkstra et al., 1984), this hydrolysis rate is probably too slow for a destabilization system. An alternative pH sensitive derivative, the citraconyl group, is hydrolyzed from citraconyl-lysine at a more rapid rate (complete in 3 hr at pH 4.2; Habeeb and Atass, 1969). An analogue of this linkage has been used by Shen and Ryzer(1981) to attach daunomycin to an inert carrier. They showed that the linkage is hydrolyzed in 3hr at pH 4. In preliminary experiments dioleoylphosphatidylethanolamine, a hexagonal-phase PE at 18^{o}C and above, was conjugated with citraconyl anhydride to prepare a citraconyl-PE. Liposomes composed of mixtures of

citraconyl-PE and PE (molar ratio 1/2) were prepared. Although about 2/3 of citraconyl groups were removed by 3hr, the liposome was found to be relatively stable at pH 4.5, releasing only 10% of the encapsulated marker after 3 hr. The stability of the hydrolyzable pH-sensitive liposomes prompted us to explore a titratable lipid, in which the protonation of the lipid at low pH would rapidly lead to a destabilization of the bilayer membrane.

Yatvin et al.(1980) reported on a liposome containing an acid-titratable lipid, N-palmitoyl-l-homocysteine. The liposome was designed for selective drug release in regions where the local blood pH is about 1 unit below normal such as sites of inflammation or tumor growth. In vitro experiments showed an enhanced release of an encapsulated aqueous marker at pH 6.0, and was attributed to the conversion of the homocysteine to a neutral thiolactone. This thiolactone was conjectured to destabilize the liposome bilayer. However, at this pH the rate of lactonization is so slow that the proposed mechanism must be seriously questioned.

Our approach to a titratable derivative is to employ hemisuccinate esters of two common membrane lipids, cholesterol and d-tocopherol(Fig. I-1). The carboxylate group of CHEMS and TS is titratable in the pH range of the endocytic vesicles(pH 4.5 - 6.0). Liposomes of PE containing more than 20 mol% CHEMS and TS are stable at neutral pH, but aggregate immediately upon exposure to a low pH medium

and release their contents(Ellens et al., 1984). Our preliminary experiments in cultured cells suggested that such liposomes deliver a polar fluorescence probe(calcein) into the cytosol. The delivery of calcein can be inhibited by the treatment of cells with ammonium chloride or chloroquine, implying that the polar probe may enter the cytoplasm in an acid-sensitive pathway. These preliminary experiments emphasized the need to understand the physico-chemical mechanisms that are involved in pH sensitive liposomes and formed the basis for studies reported in this thesis.

3. Background Information

3.1. Gel-Fluid Transition of Phospholipids

3.1.1. Phase Transitions of Phospholipid Bilayers

The transition of phospholipids from a crystalline to a liquid state on heating involves one or more intermediate liquid crystalline structures. In the presence of water, several intermediate hydrated states occur in the transition from the crystal to a suspension of phospholipid in water. Such lyotropic mesomorphism(change of state upon addition of water) also a shows temperature dependence. The structure of the phospholipid is therefore a function of both temperature and water content(Ladbrooke and Chapman, 1969; Chapman, 1975). Since phospholipids in the form of liposomes are the major interest of the present study, this discussion will be limited to the phase behavior of phospholipids in the

presence of excess water.

One method to study the phase transitions of phospholipid dispersions is to use differential scanning calorimetry. Differential scanning calorimetry of multilamellar dispersions of phospholipids with saturated acyl chains shows a narrow endothermic transition at the temperature defined as T_m(Fig. I-4). Below the phase transition phospholipids are in an ordered gel state characterized by an all-trans configuration(L_β) of the acyl chain. For synthetic saturated lipids, the hydrocarbon chains are usually packed in a hexagonal array and give a sharp X-ray reflection at 4.2Å(Tardieu et al., 1973; Franks and Lieb, 1981). The trans-gauche isomerization of the acyl chains occurs as phospholipids are heated through the transition temperature. The X-ray diffraction of saturated phospholipids above this temperature gives a diffuse spacing between 4.5Å-4.7Å which reflects the fluid state of the hydrocarbon chains(L_α). The isomerization of the hydrocarbon chains results in a 25% increase of bilayer surface area(Melchior and Morowitz, 1972) and a 10-20% decrease of bilayer thickness(Buldt et al., 1978).

A smaller endothermic peak at a temperature lower than the main transition temperature is observed with saturated phosphatidylcholines. Below this transition, PC exists in $L_{\beta'}$ form, in which the all-trans acyl chains are tilted with respect to the normal bilayer(Tardieu et al., 1973; Fig. I-

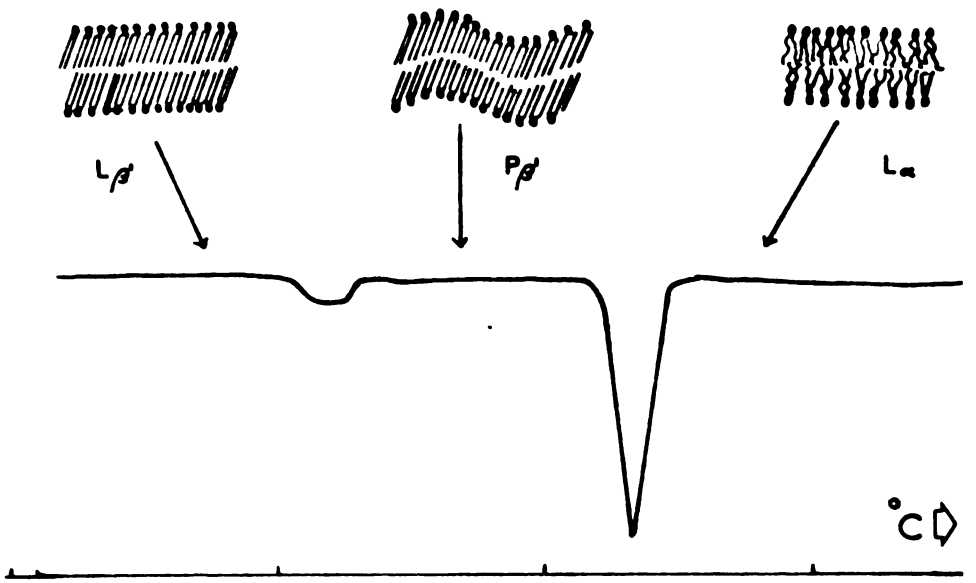


Figure I-4. Differential scanning calorimetry of the transition of phosphatidylcholine from $L_{\beta'}$, gel phase, through $P_{\beta'}$, phase, to L_{α}, liquid-crystalline phase. (Reproduced with permission, from Verleij and de Gier (1981) in "Liposomes: from Physical Structure to Therapeutic Applications", Knight, C.G. ed., Elsevier/North Holland, New York, pp 83-103)

4). During the pretransition the $L_{\beta'}$ phase undergoes a head-group reorientation into the $P_{\beta'}$ state, a phase characterized by a periodic ripple in the plane of lamella and stiff, yet still tilted hydrocarbon chains(Janiak et al., 1979). The pretransition has been attributed to the fact that the area occupied by the head-group is larger than cross-section of two acyl chains in the bilayer. Thus the hydrocarbon chains must be tilted and offset in order to increase packing in the hydrophobic region(Hauser et al., 1981). The presence of impurities or small molecules can act as fillers to expand the volume of the hydrophobic matrix to be comparable to that of head-group; the hydrocarbon chains therefore can assume a more perpendicular orientation. Thus such compounds at low concentration can inhibit the pretransition of PC(McIntosh, 1980).

Since the main transition in the lipid bilayer principally involves the melting process of the hydrocarbon chains, the transition is always correlated with properties of the acyl chains(Nagle, 1980). The transition temperature and enthalpy increase with the length of the hydrocarbon chain(see Table I-1), which is a reflection of the increased van der Waals attraction between the hydrocarbon chains. The introduction of a double bond in the hydrocarbon chain will disrupt the packing and bring about a reduced transition temperature and/or reduced enthalpy of the transition. This effect is manifest when the cis-double bond is located

in the middle of the acyl chain where a maximum destructive effect on the packing order is expected. Phospholipids containing such a double bond have been shown to have the lowest transition temperatures(Barton and Gunstone, 1975).

The head-group structure also affects the phase transition. The transition temperature of anionic phospholipids is dependent on the pH, ionic strength and the presence of divalent cations(Trauble and Eibl, 1974; Jacobson and Papahadjopoulos, 1975). The increase of T_m by the proton titration of PS and PA may be due to a enhanced intermolecular interaction between head-groups(MacDonald et al., 1976; Jain and Wu, 1977; Blume, 1979). The higher T_m of PE can be accounted for by a similar tight packing of the head-groups(discussed in 3.2.1). Thus modifications of the PE head-group, such as progressive methylation toward PC(Casal and Mantsch, 1983), significantly reduce the transition temperature.

The sharpness of the phase transition of highly purified saturated phospholipids, and the effect of pressure on the transition temperature, support the idea that the phase transitions of phospholipid are first-order transitions(Lee, 1983). However, the finite width of the lipid phase transition and the continuous volume change during the transition are not consistent with a first-order transition which in theory should be discontinuous at the transition temperature(Lee, 1977a). Lee(1977a, 1983) suggests that the

physical imperfections in the bilayer membrane, and the interfacial lipid between gel and fluid phases can account for the observed deviation from an ideal first-order transition. The structural defects serve as nuclei for localized lipid melting and thus the cooperative unit will be smaller than the whole bilayer. The simultaneous presence of a number of transition domains, which are not highly dependent on each other, will result in a transition with a finite width. Lipid phase transitions also differ from other first-order transitions in that gel and fluid phases may coexist. Unlike transitions between distinct phases such as a liquid and a gas, a significant amount of lipids are present in the interphase between the gel and fluid phases. Such interfacial lipid surrounding fluid domains can be detected by ESR(Marsh et al., 1976). This implies that the lipid melting process is not a simple equilibrium between gel and fluid phases, and that the interfacial lipid contributes to the continuous properties of the transition.

An alternative explanation for this non-ideal behavior is that a slow-relaxing, non-equilibrium distribution of domains can be generated by the presence of six or more intermediate acyl chain conformational states that occur during the phase transition(Mouritsen, 1983). This explanation does not require structural defects or impurities in the gel state to account for the width of the transition. Regardless of the interpretation of the detailed molecular

events accompanying the phase transition, differential scanning calorimetry is a valuable method to detect alteration in the phase behavior in multicomponent mixtures of lipids.

3.1.2. Phase behavior of binary lipid mixtures

In the case of binary lipid mixtures the phase rule can be applied since the phase transition of each lipid can be considered to undergo a first order change. The interaction between two components can be deduced from the phase diagram, which is constructed from the onset and completion of the transitions from a series of mixtures. The phase diagrams for a number of phospholipid mixtures have been established from DSC data(Philips et al., 1970; Mabrey and Sturtevant, 1976) and the mixing behavior is found to be dependent on the heterogeneity between the head-groups and the acyl chains. Phase diagrams of phospholipids mixtures showing eutectic behavior(Fig. I-5a) are not very common. DMPC/DPPC or DPPC/DSPC mixtures have a phase diagram which indicates a continuous gel solution(Fig I-5b). The observed phase diagram deviates only slightly from ideal mixing calculated by regular solution theory, and the non-ideality has been attributed to the van der Waals interactions of unlike hydrocarbon chains(Cheng, 1980). Deviations from ideal mixing increase for mixtures containing lipids of different head-groups, or of acyl chains with more than a four carbon unit difference. A monotectic behavior with no solid solution(Fig. I-5c) is observed in mixtures of lipids with

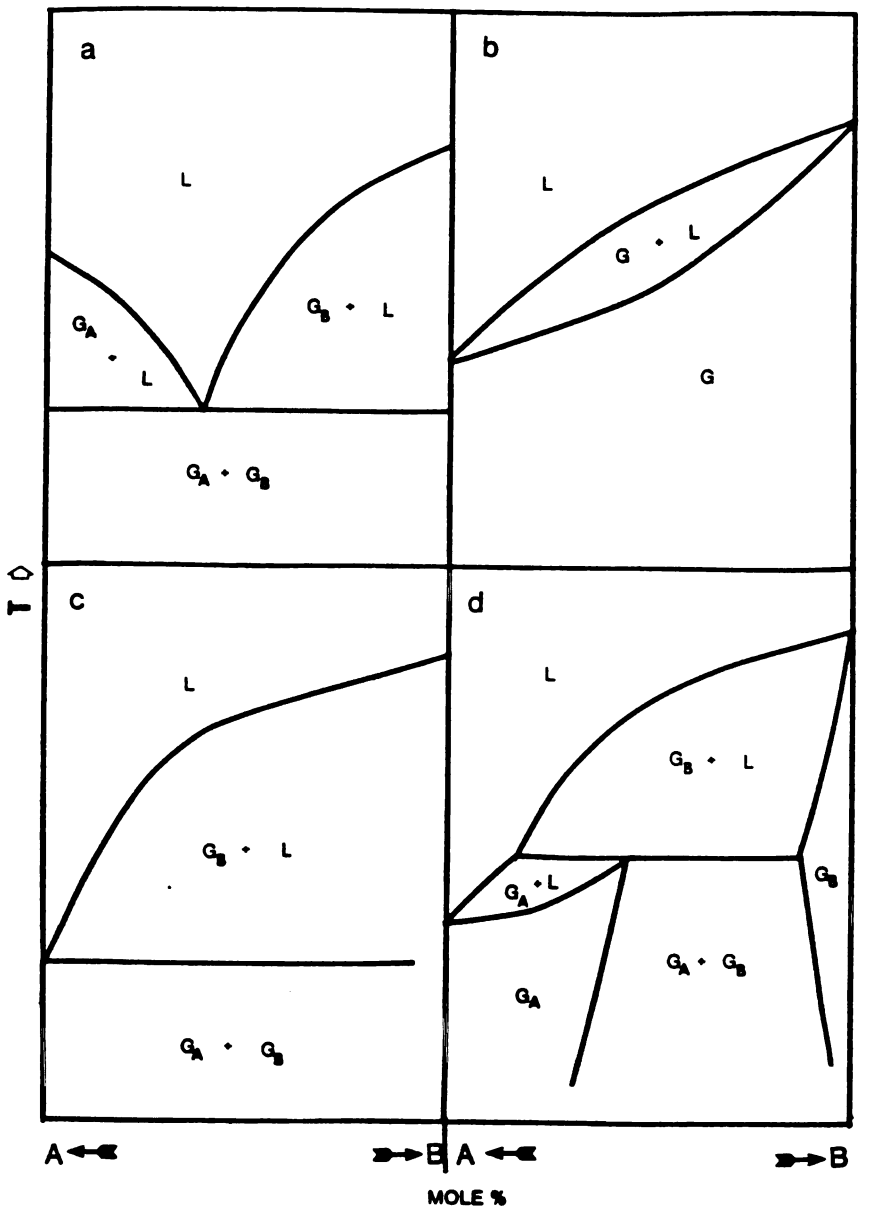


Figure I-5. Binary phase diagrams of (a) eutectic behavior, no gel-state miscibility. (b) mixtures with continuous miscibility of both gel and liquid crystalline states. (c) monotectic behavior, no gel-state miscibility. (d) peritectic behavior, partially miscible gel states. Liquid crystal phase(L) of A and B is assumed to be completely miscible.

acyl chain length differences of six carbons such as DLPC/DSPC(Mabrey and Sturtevant, 1976).

The thermotropic behavior of binary mixtures of phospholipids and other hydrophobic ligands is also determined by their interactions. Long chain fatty acids or alcohols(C12-C18) incorporated into PC(C14,C16) bilayers increase the transition temperature and eliminate the pretransition(Eliasz et al., 1976; Jain and Wu, 1977). Below the limit of phase separation(80 mol%), stearoyl alcohol and DPPC form a complex that exhibits partial gel phase immiscibility(Eliasz et al., 1976). The specific effect of fatty acids(alcohols) may be explained by the ability of fatty acid(alcohol) molecules to fit into the phosphatidylcholine lattice. The diverse effects of a number of ligands(detergents, organic solvents, ionophores etc) on the thermal properties of phospholipids may also be interpreted by their interactions with different regions of the phospholipid molecule(Jain and Wu, 1977). Although many mixtures can be understood in terms of ideal solution theory, there are phase behaviors of the binary mixtures that are still very difficult to explain(Lee, 1977b). For example, the addition of tocopherol acetate to DPPC lowers the onset temperature, but not the completion temperature of the gel-fluid transition(Schmidt et al., 1976).

The ability to detect different phases in mixtures of phospholipids by freeze-fracture electron microscopy or

other techniques has revealed more complicated features of their phase diagrams. In the DPPC/DPPE phase diagram there are regions representing the $P_{\beta'}$ phase of DPPC located in the low concentration end of DPPE(Blume et al., 1982b). A more detailed study of DMPC/DSPC indicated that this mixture exhibits peritectic behavior similar to Fig. I-5d(Knoll et al., 1981). Phase diagrams of a very complicated peritectic type have been shown for DPPC-palmitic acid and DPPC-stearic acid mixtures(Schullery et al., 1981). It may be noted that fluid phase immiscibility can be identified and included in the phase diagram(Wu and McConnell, 1975). Fluid-fluid immiscibility in the region above the liquidus line has also been detected, for example in DMPC/DSPC(Knoll et al., 1981) and in DMPC with a low content of cholesterol(Recktenwald and McConnell, 1981). Thus certain detailed molecular interactions may not be revealed by studying the phase diagram of the binary mixture by differential scanning calorimetry. Therefore both ^{31}P-NMR and freeze-fracture electron microscopy have been used in our work to detect phases that cannot be observed by DSC.

3.1.3. Thermal Properties of Phospholipid-Cholesterol Mixtures

Binary mixtures of phospholipids and cholesterol have been extensively studied(Demel and de Kruijff, 1976; Lee, 1977b) due to the unique properties of the mixtures, and because of the importance of cholesterol in biomembranes.

It is well known that the incorporation of cholesterol in membranes inhibits the main transition of the phospholipids, reduces the membrane permeability, increases the membrane microviscosity(Demel and de Kruijff, 1976), decreases the depth of water penetration into the bilayer(Simon et al., 1981), and enhances the stability of liposomes in biological fluids(Kirby et al., 1980), to name just a few of the effects of cholesterol on bilayer membranes. Recent studies using high sensitivity DSC have revealed more detailed aspects of the thermotropic behavior of phospholipid-cholesterol mixtures(Estep et al., 1978; Mabrey et al., 1978; Blume, 1980). The pretransition of DPPC is suppressed with 3.6 mole% cholesterol(Estep et al., 1978). Below 20 mole% cholesterol the endothermic curve of PC can be resolved into two components, a sharp transition at lower temperature and a broad peak at high temperature(Estep et al., 1978; Mabrey et al., 1978). As the cholesterol content exceeds 20 mole% only a broad transition is detectable which is continuously shifted to higher temperatures as the mole fraction of cholesterol increases. The transition is completely suppressed at 50 mole% cholesterol.

Most aspects of the thermotropic behavior of phospholipid-cholesterol mixtures can be explained by the model proposed by Presti et al.(1982). In membranes containing low concentrations of cholesterol, phospholipids form a complex with cholesterol in a 2:1 stoichiometry(Fig.

I-6). One phospholipid is specifically bound and the second one is loosely associated with the cholesterol. Cholesterol rich domains coexist with free phospholipid, contributing to the resolvable broad and sharp transition observed in the DSC scan. The disappearance of the sharp transition beyond 20 mole% cholesterol implies that the phospholipid is either associated with cholesterol or in a boundary layer surrounding cholesterol-rich domains. The amount of interfacial boundary phospholipid decreases as additional cholesterol is incorporated into the membrane and disappears at 33.3 mole% cholesterol. Any subsequently added cholesterol forms a 1:1 complex with the loosely bound phospholipid; thus at 50 mole% cholesterol the last phospholipid forms a complex. At this composition the phospholipid is completely isolated from other phospholipid molecules and hence a transition due to interaction between phospholipids is no longer measurable by DSC. Further addition of cholesterol results in the phase separation of hydrated cholesterol crystals from the bilayer(Lecuyer and Derichau, 1969). This model agrees well with other physical measurements(Recktenwald and McConnell, 1981; Hui and He, 1983; and references cited in Presti et al., 1982).

The formation of the phospholipid-cholesterol complex is attributed to the specific configuration of cholesterol that allows it to fit into the phospholipid lattice. The flat surface of the α face may be packed tightly with the

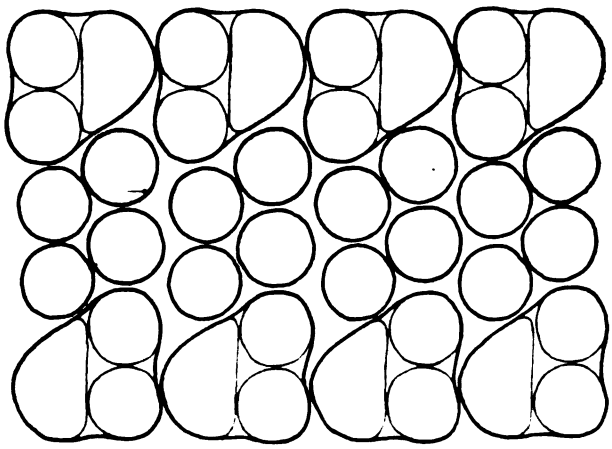


Figure I-6. Model for a domain of 1:2 cholesterol-phospholipid stoichiometry. The hydrocarbon chains of a phospholipid molecule are represented by a pair of circles. Cholesterol is depicted by the elongated shape. The 1:1 complex of cholesterol-phospholipid is enclosed by the continuous line. (Presti et al. Biochemistry 21, 3831-3835; reproduced with permission from Biochemistry, copyright 1982 American Chemistry Society))

saturated hydrocarbon chain region of phospholipid to allow a maximum van der Waals interaction(Huang, 1977; Presti et al., 1982). This interaction seems to be reduced by the increased unsaturation of the acyl chain, as indicated by the gradually diminished effect of cholesterol on the microviscosity and phase transition of PC with 1-saturated-2-unsaturated and 1,2-unsaturated acyl chains(Guyer and Block, 1983; Davis and Keough, 1983). The hydrocarbon side chain of cholesterol is also important for hydrophobic interaction; no effect is found for steroids lacking the side chain(de Kruijff et al., 1972). Of a more intriguing and controversial nature is the role of the 3β-OH group which constitutes the small hydrophilic part of cholesterol. The 3α-OH isomer of cholesterol(epicholesterol) and ketosteroids are unable to reduce the membrane permeability and suppress the phase transition of phospholipid bilayers(de Kruijff et al, 1972; Bittman et al., 1981). The incorporation of epicholesterol up to 30 mole% reduces the transition enthalpy of the phospholipid, but the extent of the reduction is small when compared to cholesterol. Increasing the epicholesterol beyond 30 mole% has no further effect on the transition of phosphatidylcholine. Substitution of the 3β hydroxyl group with a thiol group also significantly reduces the membrane ordering effect of cholesterol, and the maximum amount of cholesterol which may reside in phospholipid bilayers(Parkes et al., 1982). The partitioning of cholesterol into phospholipid bilayers is also impeded if a

3-α-methyl group is introduced into the molecule together with the β-OH(Dahl, 1981). These studies have suggested that some specific interaction between the 3β-OH of cholesterol and a polar element of the phospholipid is necessary for the complex formation between the two lipids. However, the effect of cholesterol on the permeability and phase transition of phospholipids is the the same for diacyl, dialkyl and diether PC(Bittman et al., 1981). Since the carbonyl oxygen is absent on both diether and dialkyl PC's, and the glycerol oxygen is absent on dialkyl PC, it is unlikely that the carbonyl oxygen or glycerol ester oxygen of the phospholipid is involved in intermolecular hydrogen bonding with the 3β-OH of cholesterol. Moreover the phosphate group of PC does not interact with the 3β-OH of cholesterol, as indicated by the measurement of $^{31}P(^{1}H)$ nuclear Overhauser effect(Yeagle et al., 1975). The studies presented in this thesis with the succinate ester of cholesterol also indicate that the 3β-OH is not essential for the interaction of cholesterol with phospholipids.

3.2. Phosphatidylethanolamine and the Hexagonal Phase

3.2.1. Phosphatidylethanolamine

The physicochemical properties of phosphatidylethanolamine are different from most of other phospholipids. At neutral pH the hydration of PE is very slow(Jendrasiak and Hasty; 1974), and does not result in bilayer

vesicles(Papahadjopoulos and Miller, 1967; Kolber and Haynes, 1979). Neither does PE form small unilamellar vesicles upon prolonged sonication unless the pH is increased to around 9.0(Litman, 1973; Stollery and Vail, 1977). The gel-fluid transition temperature of PE is always 15-20^{o}C higher than that of PC with the same acyl chain composition. However the most unusual aspect of PE is that it undergoes an additional transition from the bilayer phase to an inverted hexagonal phase at elevated temperature.

The atypical properties of PE have been attributed to the conformation of the head-group. The phosphorylethanolamine group is oriented parallel to the plane of bilayer; the folded head-group is stablized by a strong interaction between the ammonium nitrogen and the phosphate oxygen of the adjacent PE(Hitchcock et al., 1974; Hauser et al., 1981). In addition, the two phosphate oxygens of each molecule interact with two ammonium groups, and the ammonium group of each molecule forms hydrogen bonding with the phosphate groups of two adjacent PE (Hauser et al., 1981; Fig. I-7). This produces a very compact, rigid head-group network at the bilayer surface. Harlos(1978) has demonstrated that below the transition temperature hydrated PE has the same head group area(~39$\AA^2$) as in the crystal state. Such a direct PO---N interaction is sterically impossible in PC because of the fully methylated quaternary ammonium group(Parson and Pascher, 1979; Hauser et al., 1981).

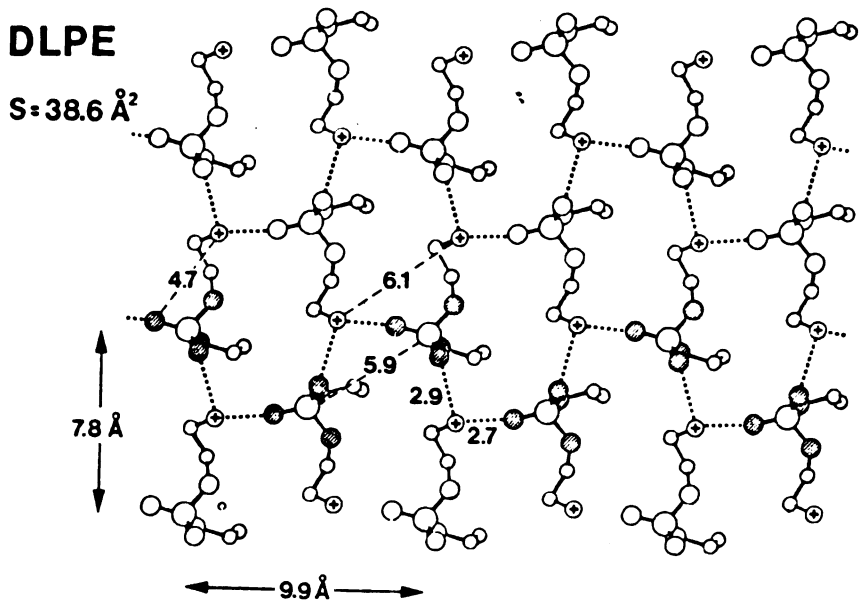


Figure I-7. Head-group packing of dilauroylphosphatidylethanolamine (DLPE) viewed from the top of the bilayer. Hydrogen bond linking phosphate and ammonium group is indicated by dotted line. The number associated with the broken line denotes the distances between atoms. The ammonium group of each molecule is interacting with two adjacent phosphate groups, and the two phosphate oxygen of each molecule are hydrogen bonding to two adjacent ammonium nitrogens. The formation of phosphate ribbon is visualized by using hatched phosphate oxygen. (Reproduced from Hauser et al. (1981) Biochim. Biophys. Acta 650, 21-51 with permission of Dr. Hauser)

This tightly interacting head-group region has a profound effect on the structural morphologies that the lipid can assume. Since the phosphorylethanolamine group displays little flexibility with temperature(Gally et al., 1980; Mantsch et al., 1981; Blume et al., 1982), the expansion of the hydrocarbon chain matrix, resulting from the increase in the population of gauche isomers at elevated temperatures, transforms PE from a cylindrical molecule into a conically-shaped molecule. The wedged shape of PE permits it to adopt a packing with a negatively curved surface which is termed an inverted hexagonal phase(Israelachvili et al., 1980; Cullis and de Kruijff, 1979; Fig. I-8). Thus, as the temperature is increased, PE in a bilayer phase can convert into PE in a hexagonal phase. Similar lamellar-hexagonal transitions are observed with molecules that can be transformed into a conical-shape, such as Ca^{++}-cardiolipin (Vail and Stollery, 1979), monoglucosyldiglyceride (Wieslander et al., 1980), and monogalactosyldiglyceride (Quinn and Williams, 1983).

The inability of PE to form stable bilayer vesicles below its hexagonal transition temperature is also related to its head-group orientation. The electrostatic free energy of PE lattice is considerably less than that of PC lattice(Hauser et al., 1981) because of the extensive interaction between head-groups in the same plane. The repulsion between PE head-groups is quite low which permits

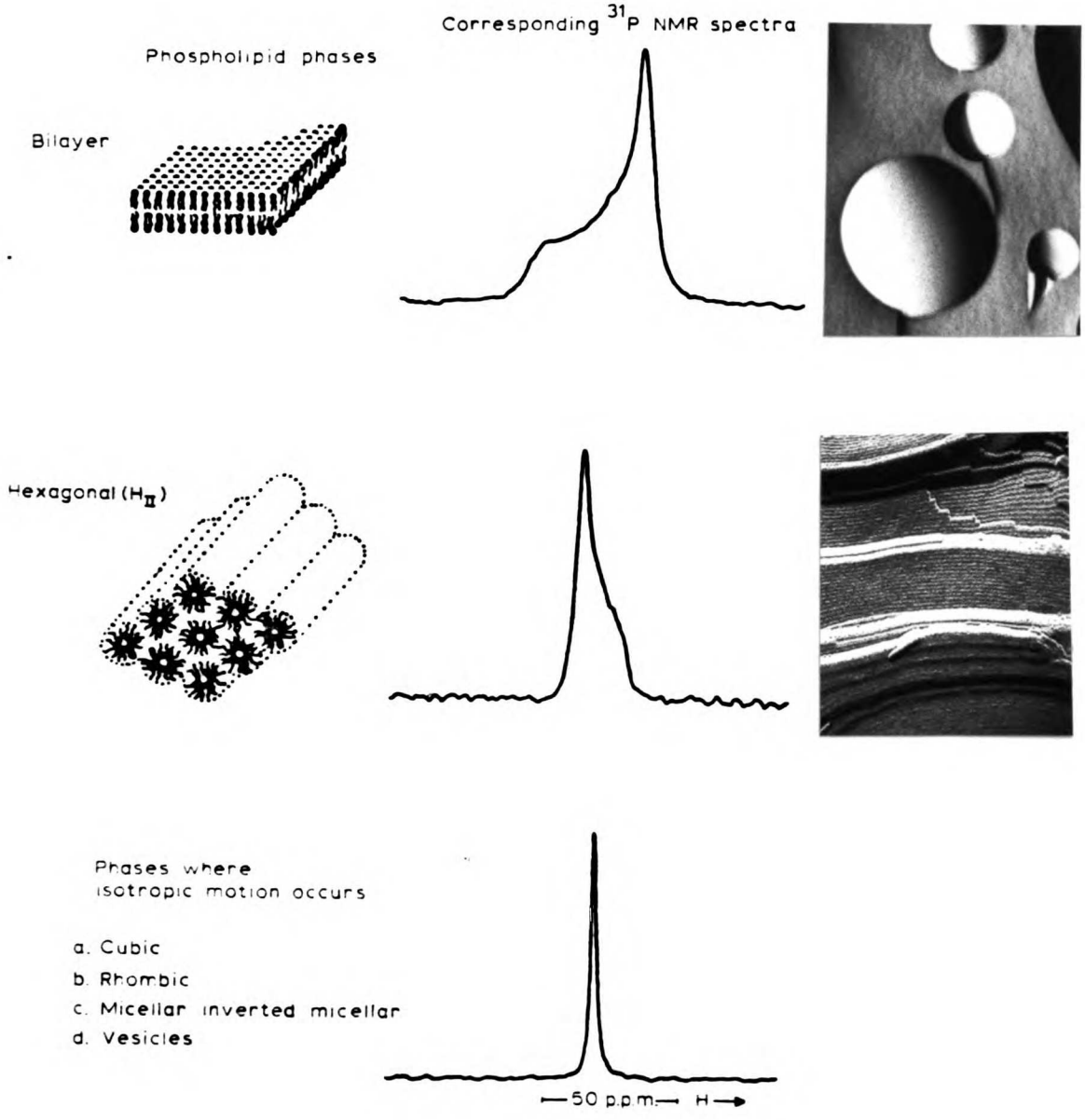


Figure I-8. Three different types of phospholipid phases, their freeze-fracture electron micrographs, and the corresponding ^{31}P-NMR spectra. (Phospholipid phases and ^{31}P-NMR from Cullis and de Kriijff, 1978; Reprinted by permission from Nature, Vol. 271, No. 5646, pp 672-674, copyright (c) 1978 Macmillan Journals Limited)

the close contact of separate bilayers. This results in the aggregation of PE bilayers. In addition, the ratio of the head group area to the cross section of the hydrocarbon chain matrix, which determines the packing pattern of amphiphiles, is close to 1 for PE in the lamellar phase. Thus on theoretical grounds a planar bilayer would be the structure preferred by PE instead of a curved bilayers(Israelachvili et al., 1980). PE vesicles can be prepared at an alkaline pH where the amino group is deprotonated but as the pH is neutralized they will flatten into a disc like structure(Stollery and Vail, 1977). The ability of PE to form either a lamellar phase or hexagonal phase depending on pH may be biologically important and gives one the capacity to prepare membranes that respond to their environments.

3.2.2. Detection of the Hexagonal Phase

a. Freeze-fracture electron microscopy

Inverted hexagonal structure can be directly visualized by freeze-fracture electron microscopy. In this technique a specimen is rapidly frozen in liquid nitrogen(or liquid freon, propane). The fracture plane always runs between CH_3 groups of the acyl chains of the phospholipid. The fractured specimen is then replicated by shadowing with platinum/carbon followed by carbon shadowing to increase the mechanical stability for microscopic examination. The fracture face of the hexagonal structure is composed of long parallel cylinders which occurs along at least two fracture

planes(Fig. I-8). The lipid cylinder has a diameter of 50Å-70Å with an aqueous core of 20Å. This type of fracture face is distinctive from the smooth fracture face obtained with bilayer vesicles(Fig. I-8).

b. Differential scanning calorimetry

Bilayer-hexagonal transitions of saturated PE's and some naturally-occurring PE's can be detected by differential scanning calorimetry(Harlos and Eibl, 1981; Boggs et al., 1981; Seddon et al., 1983). The enthalpy associated with the hexagonal transition is small when compared with the gel-fluid transition, indicating that the energy barrier between the lamellar and hexagonal phase is low(Cullis and de Kruijff, 1979; Boggs, 1981). This is manifest with synthetic unsaturated PE's, in which the hexagonal transition is not detectable by DSC(van Dijck et al., 1976). The L - H transition commonly occurs over a 5-10°C temperature interval for both synthetic and naturally-occurring PE(Cullis and de Kruijff, 1978a; Harlos and Eibl, 1981). Other physical measurements also show a similar L - H transition range.

c. ^{31}P-NMR

^{31}P-NMR has been extensively used to study the motion and average orientation of phosphate groups of membrane phospholipids(Seelig, 1978). Phospholipids in different membrane structures can be distinguished by their ^{31}P-NMR spectrum(Cullis and de Kruijff, 1979; Fig. I-8). For multilamellar vesicles, the chemical shift anisotropy of lipid

phosphorous is only partially averaged due to the restricted motion of the phospholipids in the bilayer. With proton-decoupling to remove proton-phosphorous dipolar interactions, a characteristic broad spectrum with a low field shoulder and a high field peak is generated. In the case of small unilamellar vesicles, the rapid lateral diffusion of phospholipids within the plane of the vesicle effectively averages out the phosphorous chemical shift anisotropies, and results in a spectrum consisting of a narrow peak. The spectrum of the hexagonal phase shows reversed asymmetry relative to the bilayer spectrum, and a two-fold reduction in the linewidth as the result of motional averaging effects around the small aqueous channel in H_{II} phase.

Recently there have been questions raised about the interpretation of this type of spectrum. In a theoretical calculation, Thayer and Kohler(1981) showed that the spectrum associated with the bilayer, hexagonal or isotropic structures can be generated by changing the phospholipid head-group conformation while retaining molecules in the bilayer structure. Hui et al.(1980, 1981a) reported that electron microscopy and X-ray diffraction failed to detect the hexagonal phase suggested by ^{31}P-NMR spectrum in the dispersions of sphingomyelin(1980) and soybean PE/egg PC mixture(1981a). In the latter case, they proposed that the NMR signal might come from lipidic intramembrane particles that would allow the PO_4 to assume a more isotropic orienta-

tion. Bilayers of phosphatidyldiacylglycerol and phosphatidylcholesterol also generate ^{31}P-NMR spectrum characteristic of hexagonal and isotropic phases(Noggles et al., 1982). These finding necessitate that caution must be taken on interpreting ^{31}P-NMR spectrum. However, the technique is still helpful in differentiating phase polymorphism when used in conjunction with other physical measurements and electron microscopy.

d. Other physical measurements

The X-ray diffraction pattern of phospholipids in the H_{II} phase give rise to long spacing in the low angle region which is characteristic of the hexagonal packing of the lipid cylinder. This is in sharp contrast to the diffraction pattern of the lamellar structure which gives a long spacing with a completely different ratio (Harlos and Eibl, 1981; Marsh and Seddon, 1982; Seddon et al., 1983).

Deuterium-NMR can be used to measure the increased disorder of the deuterium labeled acyl chain of PE when it is in the hexagonal phase. In the temperature region of the transition the intensity of lines with quadrupole splitting characteristic of the lamellar phase decreases, and a new pair of lines with a smaller quadrupole splitting appears on the spectrum(Gally et al., 1980). Similar changes of ^{2}H-NMR can be observed with head group-deuterated PE during the L - H transition(Taylor and Smith, 1981).

The increase in conformational disorder of acyl chains accompanied with hexagonal phase formation can be detected by spin label electron spin resonance spectroscopy(Hardman, 1982). In the temperature region of the L - H transition, a decrease in the central line width of the ESR spectrum, and a decrease in the order parameter are observed. As one would anticipate, the change is more prominent as the nitroxide group is situated toward the end of the acyl chains(Hardman, 1982). Infrared spectroscopy can also be used to detect the hexagonal phase(Mantsch et al., 1981). The IR spectrum originating from the acyl chain (methyl, methylene, CH=CH) and from the ester linkage (C=O, C-O) is altered by the hexagonal transition(Mantsch et al., 1981). Both methods indicate an increase in the population of gauche isomers in the formation of the hexagonal phase.

Table I-1 lists a number of synthetic and natural-occurring phosphatidylethanolamines with which the bilayer-hexagonal transition has been identified by the methods described above.

3.2.3. Factors Affecting the Bilayer-Hexagonal Transition

a. Acyl chain region

The hexagonal transition is enhanced by the increase in the volume of the hydrocarbon chain matrix. Table I-1 display a good correlation of acyl chain length with T_m and T_h. Gel-fluid transition temperatures increase with the

TABLE I-1

Hexagonal Transition Temperature of Phosphatidylethanolamines*

I. Synthetic Phosphatidylethanolamines

a. Saturated Phosphatidylethanolamines - Acyl Linked

Species	T_m	T_h	Detected By	Ref
12:0/12:0(water)		>150°C	DSC, X-ray	a
12:0/12:0(pH 7.0)		>90°C	P31-NMR	b
14:0/14:0(water)		>150°C	DSC	a
14:0/14:0(pH 7,1M NaCl)	52°C	ND[1]	X-ray, DSC	c
14:0/14:0(6.25M NaCl)[2]	57°C	97°C	DSC, X-ray	a
16:0/16:0(water)	64°C	123°C	DSC, X-ray	a
16:0/16:0(pH 7,1M NaCl)	62.5°C	ND	DSC, X-ray	c
16:0/16:0(pH 7,4M NaCl)	70.5°C	92°C	DSC, X-ray	c
16:0/16:0(6.25M NaCl)	71°C	86°C	DSC, X-ray	a
18:0/18:0(water)	74°C	101°C	DSC, X-ray	a
18:0/18:0(pH 7,1M NaCl)	77°C	101.5°C	DSC, X-ray	c
18:0/18:0(pH 7,2M NaCl)	78°C	95.5°C	DSC, X-ray	c
18:0/18:0(pH 7,4M NaCl)	80°C	88.5°C	DSC, X-ray	c
18:0/18:0(6.25M NaCl)	78°c	78.4°C	DSC, P31-NMR	d
20:0/20:0(water)	82.5°C	96°C	DSC, X-ray	a
20:0/20:0(6.25M NaCl)	83°C	83°C	DSC, X-ray	a

*see last page of table for notes.

TABLE I-1 (continue)

b. Saturated Phosphatidylethanolamines - Ether Linked

Species	T_m	T_h	Detected By	Ref
12:0/12:0(water)	35°C	110°C	DSC, X-ray	a
12:0/12:0(6.25M NaCl)	45°C	73°C	DSC, X-ray	a
14:0/14:0(water)	55.5°C	96°C	DSC, X-ray	a
14:0/14:0(pH 7,1M NaCl)	58°C	93.5°C	DSC, X-ray	c
14:0/14:0(6.25M NaCl)	64°C	75°C	DSC, X-ray	a
16:0/16:0(water)	68.5°C	86°C	DSC, X-ray	a
16:0/16:0(pH 7,10mM NaCl)	69.1°C	88°C	DSC, P31-NMR	e
16:0/16:0(pH 12.3)	45.6°C	ND	DSC	e
16:0/16:0(pH 7,1M NaCl)	71.5°C	87°C	DSC, X-ray	c
16:0/16:0(6.25M NaCl)	74°C	74°C	DSC, P31-NMR	d
16:0ether/16:0ester	66°C	102°C	DSC, P31-NMR	e
18:0/18:0(water)	77°C	87°C	DSC, X-ray	a
18:0/18:0(6.25M NaCl)	82°C	82°C	DSC, X-ray	a

TABLE I-1 (continue)

c. Unsaturated Phosphatidylethanolamines

Species	T_m	T_h	Detected By	Ref
16:1c/16:1c	-35°C	0°C	EM	h
16:0/18:1	28°C	75°C	P31-NMR	i
18:1t/18:1t	38°C	55°C	P31-NMR	b
18:1c/18:1c	-16°C	10-15°C	P31-NMR	f
18:1c/18:1c		10-15°C	H2-NMR	g
18:1c/18:1c		20°C	EM	h
18:2/18:2		-10°C	P31-NMR	j
18:3/18:3		-30-0°C	P31-NMR	i
20:4/20:4		<-30°C	P31-NMR	i
20:6/20:6		<-30°C	P31-NMR	i

TABLE I-1 (continue)

II. Natural Phosphatidylethanolamines

Species	T_m	T_h	Detected By	Ref
egg PE	11.3°C	32-45°C	DSC, P31-NMR	e
egg PE	12°C	~32°C	DSC, P31-NMR	b
egg PE	12°C	28°C	FT-IR	k
egg PE		~35°C	X-ray	l
egg PE	11°C	28.5°C	DSC, ESR	m
egg PE(pH> 8.5)	10°C	ND	DSC, ESR	m
TPE(egg PC trans.)	19.8°C	63°C	DSC, P31-NMR	e
TPE	18°C	50°C	FT-IR	k
human erythrocyte PE	~10°C	~10°C	DSC, P31-NMR	b
E. Coli PE	62°C	62°C	P31-NMR	b
soy bean PE		-30°C	P31-NMR	n
rat liver microsomal PE		5°C	P31-NMR	o
plasminogen	3°C	18°C	P31-NMR, DSC	e

1. ND: not detectable.
2. 6.25M NaCl is saturated saline.
3. References: (a) Seddon et al., 1983. (b) Cullis & de Kruijff, 1978a. (c) Harlos & Eibl, 1981. (d) Marsh & Seddon, 1982. (e) Boggs et al., 1981. (f) Cullis & de Kruijff, 1976. (g) Tilcock et al., 1982. (h) van Dijck et al., 1976. (i) Dekker et al., 1983. (j) Cullis et al., 1982. (k) Mantsch et al.,1981. (l) Reiss-Hussion, 1967. (m) Hardman, 1982. (n) Cullis & de Kruijff, 1978b. (o) de Kruijff et al., 1980.

length of the acyl chain, while T_h shows a reversed trend. The T_m and T_h of long-chain PE converge in the presence of high NaCl concentrations, and in this case PE can be transformed directly from a lamellar gel phase to a hexagonal phase without passing through a liquid crystalline phase(Marsh and Seddon, 1982).

The introduction of double bonds into the hydrocarbon chain expands the acyl chain region of PE and hence can lower the L - H transition temperature. For instance DSPE remains in the lamellar phase until 100°C, whereas the L - H transition of dielaidoyl PE(18:1t/18:1t) occurs at 55°C. The more drastic disruption of the hydrocarbon chain packing by cis double bonds decreases the T_h of DOPE(18:1c/18:1c) to 15°C. The T_h of the corresponding PE with a second double bond, dilinolenyl PE(18:2/18:2), is -10°C. A similar correlation is found with naturally-occurring PEs in which the acyl chain composition is heterogeneous. Egg phosphatidylethanolamine(EPE) and transesterified egg phosphatidylethanolamine from PC(TPE) both have a similar composition of the saturated acyl chain in position 1. But the T_h of EPE is 30°C lower than that of TPE because EPE contains a high proportion(16%) of acyl chains with multiple sites of unsaturation(22:6) (Mantsch et al., 1981).

The addition of ligands into PE in the lamellar phase, that increase the cross sectional area of the acyl chain region more than the head-group region, promote H_{II} forma-

tion. Mixtures containing equimolar cholesterol and PE have been shown to have a T_h 10-20^{o}C lower than pure PE(Dekker et al., 1983). The ability of steroids to promote the H_{II} phase increases with the hydrophobic molecular area of each steriod in monolayers(Gallay and de Kruijff, 1982). In a binary mixture of DOPE-DOPC, the addition of 2 mole% cholesterol promotes the formation of the hexagonal phase(Tilcock et al., 1982). Since the cholesterol by itself does not discriminate between PC and PE(Blume, 1980; Tilcock et al., 1982), the H_{II} promotion is not a result of a phase segregation, but rather of the ability of cholesterol to expand the acyl chain region more than it does the head-group region of the bilayer.

In certain cases the incorporation of exogenous ligands into PC bilayers may produce the hexagonal phase. PC is known for its preference to be in a lamellar phase over a wide range of temperatures and hydration states(Hauser et al., 1981). However the addition of 66 mole% palmitic acid transforms DPPC directly from a lamellar gel to a hexagonal phase at 61^{o}C at or below pH 4.0 where the palmitic acid is protonated(Marsh and Seddon, 1982).

b. Head group area

Since it is the ratio of head group to acyl chain areas that dictate which structural phase an ensemble of self associating molecules will adopt, treatments that can reduce the head-group area can promote hexagonal phase formation.

Table I-1 illustrates the effect of NaCl on the L - H transition of PE. The increase in NaCl concentration raises the main transition temperature but decreases the hexagonal transition temperature of PEs. Increasing salt concentrations also sharpen the H_{II} transition and increase the enthalpy associated with the transition(Harlos and Eibl, 1981; Seddon et al., 1983). Divalent and polyvalent cations enhance the hexagonal transition at a much lower concentration than do monovalent cations. 2.0 mM Zn^{++} or 1.0 mM Cr^{+++} precipitate EPE into mixtures of lamellar and hexagonal structures, while 30mM $BeCl_3$ transforms EPE into aggregates predominately in the H_{II} phase(Stollery, 1977). The effects of salts on promoting the H_{II} phase are believed to be the result of a partial dehydration of the PE head-group. This occurs either by a direct displacement of water of hydration or by interfering with the interaction of water molecules with the head group. The decrease in the L-H temperature by salt is similar to that observed when head-group hydration is lowered by decreasing the water content of the lipid dispersion(Seddon et al., 1983).

The lower T_h of the ether-linked PE's compared to those of ester-linkage(Table I-1) may also be due to a change in the head-group interactions. Although the fine structure configuration of ether-linked PE is still unknown, Boggs et al.(1981) suggest that the packing of ether-PE increases the intermolecular head-group interaction to a small extent.

The increase in head-group interaction is reflected by a slight increase in T_m. The enhanced head-group interaction is proposed to bring about a hexagonal transition at lower temperature.

On the other hand, interference of the specific head-group interactions between adjacent PEs will increase the flexibility and water content of individual phosphorylethanolamine groups, resulting in an increased polar group area. The expanded polar head area is then comparable to the cross sectional area of the acyl chains, and PE can form stable bilayer membranes even at high temperature. Modification of the head-group by deprotonation of the ammonium group at alkaline pH(Table I-1, Cullis and de Kruijff, 1978; Hardman, 1982), methylation of the amine group, or increasing the distance between the phosphate and amine group(Seddon et al., 1983) inhibit the formation of the hexagonal phase. The incorporation of exogenous amphiphiles with a proper polar head group can also stablize PE in the bilayer structure by interrupting the interaction between adjacent PE's. Mixtures of soybean PE with 30mole% egg PC will form a stable lamellar phase even at 50°C(Hui et al., 1981). Other phospholipids such as PS(Tilcock and Cullis, 1981), sphingomyelin(Cullis and Hope, 1980), and PG(Farren and Cullis, 1980) display a similar bilayer stabilizing effect. In proper ratios detergents such as octylglucoside also stabilize EPE and DOPE in the lamellar

phase(Maddern and Cullis, 1982). In Chapters II and III, TS and CHEMS are shown to stabilize PE in a bilayer structure. This stabilization can be reversed when the carboxylate of the TS or CHEMS is protonated at low pH, resulting in a collapse of the bilayer into a H_{II} structure.

3.2.4. Non-Bilayer PE and Membrane Fusion

The fact that PE can exist in two quite different structures is by itself an interesting physicochemical subject. Moreover PEs comprise between 30-40% of eucaryotic membranes. Most of the PE is asymmetrically distributed in the inner leaflet of the plasma membrane. This high preponderance of PE on the inner membrane surface has suggested to some that it has a major role in endocytic and exocytic phenomena which involve fusions between membrane systems. The lamellar-hexagonal transition is the biophysical mechanism that has been proposed to be an important event in such membrane fusion(Cullis and de Kruijff, 1979).

Several intermediate phases in the bilayer-hexagonal transition have been identified. Of special interest is the observation by electron microscopy of bilayers containing particles or pits. By electron microscopy two types of particles can be identified. The first are spherical particles and pits which are either packed in an ordered cubic array or found randomly distributed in the bilayer surface. The second type of particles are of a conical nature and can exist either individually or in the form of ridges in the

plane of bilayer. The nature of these lipidic particles has been under debate and is still controversial. Recently Boni and Hui(1983) proposed that the conical particles are the attachment sites formed by hydrophobic connections between adjacent bilayers(Miller, 1980; Fig. I-9, a), and the spherical particles are inverted micelles sandwiched between monolayers of lipid(De Kruijff et al., 1979; Fig. I-9, b). Another particle structure named an inverted micellar intermediate(IMI) (Fig. I-9, c) has been proposed by Siegel(1984) as a transition structure between the bilayer and hexagonal phase. The aggregation of IMI forms an elongated inverted micelle column(Fig. I-9, d) which is the precursor of the hexagonal structure. Calculations show that closely opposed bilayers are required for IMI formation, and under certain circumstances it can form quite readily, but its half life is calculated to be very short(0.45 ms for egg PE; Siegel, 1984).

Non-bilayer structures such as lipidic particles leading to a hexagonal phase may play an important role in membrane fusion although this is a subject that is still controversial(Cullis and de Kruijff, 1979; Hui et al., 1981b; Rand et al., 1981). During membrane fusion a portion of the lipid must undergo a transient stage involving non-bilayer structure. Based on electron micrographs of fusing systems, a number of groups have suggested that the point defect structures such as lipidic particles and pits in the

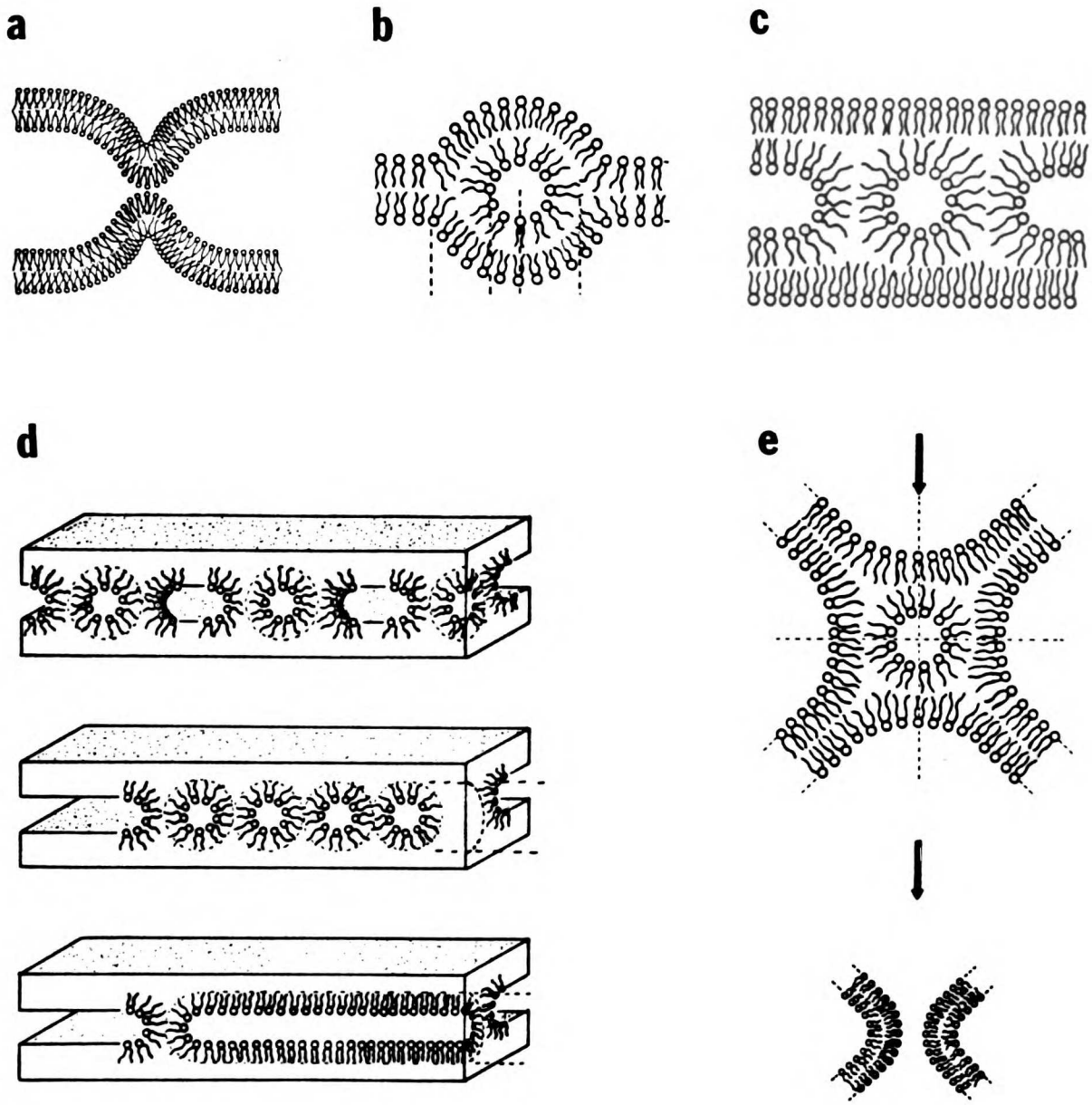


Figure I-9. Schematic diagram of (a) membrane attached site; (b) inverted micelle sandwiched in monolayers; (c) inverted micellar intermediate(IMI); (d) aggregation of IMI to from H_{II} phase: many IMI are present between apposed lamella(top), IMI aggregates form strings of inverted micelles(middle), aggregated micelles form an elongated cylindrical micelle(bottom); (e) rearrangement of micellar lipid results in membrane fusion. (a, Miller, 1980; reprinted by permission from Nature, Vol. 287, No. 5778, pp 166-167, copyright (c) 1980 Macmillan Journal limited; b-e, reproduced from Siegel (1984) Biophys. J. 45, 399-420, by copyright permission of the Biophysical Society)

bilayer may be the intermediate stage of membrane fusion(Hui et al., 1981; Rand et al., 1981). Free energy calculations indicate that lipidic particles such as the sandwiched inverted micelles and inverted micellar intermediates can only form in lipid systems with components that can form an H_{II} phase(Siegel, 1984). Figure I-9e demonstrates one scheme where membrane fusion can result from the rearrangement of the inverted micellar lipids. Such a fusion is calculated to be possible only in the temperature region of the lamellar-hexagonal transition(Siegel, 1984). Indirect evidence, which supports a role for the hexagonal phase in fusion, is the finding that the hexagonal phase can be induced in red cell ghosts by oleic acid at the same concentration that mediates fusion of erythrocytes(Cullis and Hope, 1978).

The evidence that implicates the hexagonal phase, or inverted micelles, in membrane fusion is still indirect. However it raises the possibility that lipid vesicles that can undergo a lamellar-hexagonal transition as a function of a controllable parameter such as pH might be suitable candidates for controlled drug release. In view of the fact that liposomes are internalized by an endocytic pathway, which positions them in a low pH environment, the prospect for triggering a lamellar-hexagonal transition in the liposome is one possibility for catalyzing delivery of the liposome contents into the cytoplasm of the cell.

4. Organization of the studies in the thesis

Differential calorimetry analysis, ^{31}P-NMR and freeze-fracture electron microscopy have been used to elucidate the interactions of CHEMS or TS with phospholipid membranes. The membrane structural changes that accompany the lowering of pH of mixtures of these compounds and appropriate phospholipids have also been examined. This study is divided into the following three parts. Chapter 2 investigates the interaction of CHEMS and TS with model phospholipids by studying the thermotropic behavior of the lipid mixtures. The role of the hydroxyl group on the specific interaction between cholesterol/tocopherol and the phospholipid are examined. Chapter 3 elucidates (1) the stablization effect of charged CHEMS on phosphatidylethanolamine-containing membranes, and (2) the hexagonal phase promoting effects of protonated CHEMS in the same membrane. The pH effect is compared with the charge neutralization effects of Ca^{++}. Chapter 4 examines the acid-induced structural changes that occur in TS-PC membranes. Each chapter has been written as a self contained unit to minimize the necessity of referring to other sections.

CHAPTER II

THERMOTROPIC BEHAVIOR OF PHOSPHOLIPID-CHOLESTERYLHEMISUCCINATE AND PHOSPHOLIPID-TOCOPHEROL ACID SUCCINATE MEMBRANES

1. Summary

The role of the hydroxyl groups of cholesterol and tocopherol in mediating their interaction with phospholipid bilayers has been a subject of considerable interest. We have examined this question by using derivatives of cholesterol and tocopherol in which the hydroxyl group is esterified to succinate. The hemisuccinate esters of cholesterol and d-tocopherol can be readily incorporated into phospholipid membranes and in fact can by themselves form closed membrane vesicles as demonstrated by the encapsulation of ^{3}H-sucrose. The thermotropic behavior of mixtures containing each succinate ester and phospholipid were studied by differential scanning calorimetry. The effect of cholesteryl hemisuccinate on the thermotropic properties of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylethanolamine is very similar to that of cholesterol. This indicates that the 3-β OH is not required for the formation of a cholesterol-phospholipid complex. In mixtures of tocopherol acid succinate and phospholipids the peak

transition temperature is progressively shifted to lower temperatures as the mole fraction of d-tocopherol succinate is increased, while the enthalpy of the transition is only slightly affected. At a tocopherol succinate/phospholipid molar ratio of 9/1 the phase transition of the phospholipid is still detectable. Thus the hydroxyl group of tocopherol appears to be more important than the hydroxyl group of cholesterol in influencing their interactions with phospholipids.

2. Introduction

The biological functions attributed to cholesterol and d-tocopherol have stimulated a considerable number of studies on their physicochemical properties and their interactions with phospholipids. In the case of cholesterol, up to 50 mole% can be dissolved in phosphatidylcholine membranes (Ladbrooke et al., 1968; Lecuyer and Dervichian, 1969). Below this limit cholesterol and lecithin form complexes of varying mole ratios that have been discussed in a number of recent reviews (Demel and de Kruijff, 1976; Presti et al., 1982). Although not as well studied, a complex between tocopherol and polyunsaturated phospholipids has been proposed by Diplock and Lucy (1973). Moreover, tocopherol, like cholesterol, can significantly broaden the gel - liquid crystalline phase transition in phospholipid membranes

(Massey et al., 1982) and, in the case of bilayers composed of unsaturated phospholipids, reduce the permeability of small molecules (Diplock et al., 1977).

The structural features of cholesterol that have been considered to be important for the formation of the phospholipid complex include the planar α face of the molecule, the acyl chain of between 5-7 carbons, and the β-OH group which has been suggested to participate in a hydrogen bond with a polar component of the phospholipid (Brockerhoff, 1974; Huang, 1977; Presti et al., 1982). In the case of tocopherol the hydroxyl group has been suggested to form a hydrogen bond with one of the oxygen atoms of phospholipid (Srivastava et al., 1983), while the phytanoyl chain has been considered to play a role in the tocopherol-unsaturated phospholipid interaction (Diplock and Lucy, 1973).

We had used derivatives of cholesterol and tocopherol modified at the hydroxyl group to prepare lipid vesicles which are destabilized at low pH (Ellens et al., 1984a; Lai and Szoka, unpublished results), and became interested in the question of the role of the hydroxyl group in the interaction of these compounds with phospholipids. Reports in the literature concerning a number of hydrophilic substituents of the β-OH group of cholesterol indicated that such derivatives in phospholipids membranes behave in many respects like cholesterol (Lyte and Shinitzky, 1979; Shinitzky et al., 1979; Colombat et al., 1981; Demel et al.,

1984). The increase in lipid membrane microviscosity and degree of order caused by cholesteryl phosphorylcholine (Lyte and Shinitzky, 1979) and cholesteryl phosphate (Colombat et al., 1981) is close to that induced by cholesterol. Although the crystal structures of cholesteryl phosphate and cholesteryl sulfate have been elucidated (Pascher and Sundell, 1977; 1982), little additional information is available on the interaction between charged esters of cholesterol or α-tocopherol and phospholipids. In the present study, we have demonstrated that cholesterylhemisuccinate and α-tocopherol acid succinate are incorporated into phospholipid membranes and can in fact form membranes by themselves. In its influence on the thermotropic properties of phospholipids, cholesterylhemisuccinate behaves much like cholesterol, which supports previous suggestions (Cadenhead and Muller-Landau, 1979; Demel et al., 1984) that a specific interaction between the β-OH group and the phospholipid is not necessary for the cholesterol-phospholipid complex to form. However replacing the hydroxy group of tocopherol with a succinate group dramatically changed the thermotropic properties of membranes composed of the tocopherol and phospholipid, suggesting that the hydroxyl group is an important structural feature mediating the tocopherol-phospholipid interaction.

3. Materials and Methods

3.1. Lipids and Chemicals

Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylethanolamine (DMPE) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate (morpholine salt) and d-tocopherol acid succinate were purchased from Sigma (St. Louis, MO). d-Tocopherol acid succinate was converted to its morpholine salt by mixing with equimolar morpholine(Sigma) in chloroform solution. All lipids were shown to be pure by thin layer chromatography and were stored under nitrogen at -40°C. ^{3}H-Sucrose was obtained from Amersham (Arlington Heights, IL). All other chemicals were reagent grade or better.

3.2. Preparation of Aqueous Dispersion of Lipids

Lipids (15μmole total) were deposited onto the sides of a screw cap tube (13 X 100 mm) by removing the organic solvent on a rotatory evaporator, then hydrated in 0.75 ml of 50 mM Tris buffer (pH 7.4, 100mM NaCl) with constant N_2 flushing and intermittent vortexing for at least 1 hr at a temperature 10°C above the transition temperature of the phospholipids. For DPPC/TS at 1/9 molar ratio, the dispersion was prepared by brief sonication(1 min) prior to vortexing. Lipid mixtures containing DMPE were hydrated in a buffer of pH 9.5 (carbonate-bicarbonate 25mM, NaCl 100mM) and then neutralized to pH 7.6 by concentrated Tris buffer(300mM, pH 7.0). The hydration at pH 9.5 was required for the thermograms of DMPE-TS mixtures to be reproducible.

Direct hydration of DMPE-TS at pH 7.4 occasionally resulted in split peaks on the DSC scans. The reason is still unknown. The final solution contained 20 mM bicarbonate, 60 mM Tris and 80 mM NaCl. Samples that were dialyzed against 100mM NaCl, 50mM Tris, pH 7.4 buffer showed identical DSC scans.

3.3. Differential Scanning Calorimetry

Samples for DSC were concentrated by centrifugation in an Eppendorf centrifuge(12,800g, 1 min) and the pellet was dispersed in 50 µl of buffer. 17 µl of the final dispersion was sealed in an aluminum sample pan. The lipid content in the pan was increased for preparations with a lower phospholipid/TS or CHEMS ratio. DSC measurements were made with a Perkin-Elmer DSC-2 calorimeter operating at a sensitivity of 1 mcal/sec and a scanning rate of 5^{o}C/min. At least 3 different samples were studied for each composition of the mixture except those of very low phospholipid content (2 samples were studied for DPPC/TS and DMPE/TS at 3/7, 2/8 and 1/9 molar ratios). Three heating and two cooling runs were performed on each sample. The transition enthalpy was calculated by weighing cut-outs of the peak area(heating scan), using indium as a standard. The phospholipid contents of the sample pan were determined by the method of Bartlett (1959). The transition temperatures of DPPC and DMPE, as measured by extrapolation of the rising phase of the endothermic curve to the baseline, were 41.7 $\pm$

0.2°C(N=4) and 48.6 ± 0.5°C(N=6) respectively. The enthalpy of transition was 8.7 ± 0.5 kcal/mol for the main transition of DPPC, and 6.5 ± 0.3 kcal/mol for DMPE. The results agree very well with values in the literature (Silvius, 1982). However, due to the difficulty of measuring transition temperatures accurately on a broadened transition(Eliasz et al., 1976), the temperature at the maximum of the excess heat curve was used in the present study. The peak transition temperatures for DPPC and DMPE were 42.4°C and 50.2°C respectively.

3.4. Sucrose Encapsulation

The encapsulation volume of multilamellar liposomes was determined by ^{3}H-sucrose encapsulation. After preparation of the lipid dispersion, free ^{3}H-sucrose was removed by dialysis against Tris buffer(pH 7.4, 100 mM NaCl) for two days at room temperature with several buffer changes. The buffer volume was 500 times the sample volume. A small aliquot of sample was assayed for its phosphate content and radioactivity. The encapsulation ratio was calculated by comparing the radioactivity remaining to radioactivity in the original mixture.

4. Results

4.1. Formation of Liposomes as Indicated by Sucrose Encapsulation

The succinate esters of cholesterol and d-tocopherol could be dissolved in phospholipid bilayers far beyond the limits of the parent compounds. Aqueous dispersions of mixtures of phospholipid with CHEMS or TS could be prepared easily. The measurement of ^{3}H-sucrose encapsulation of lipid mixtures indicated that the capture volumes were between 1 and 4 µl per µmole total lipid, similar to the entrapped volume of multilamellar vesicles of phospholipids (Szoka and Papahadjopoulos, 1980). Of particular interest is the capacity of DMPE-CHEMS and DMPE-TS to encapsulate sucrose, in contrast to the difficulties of preparing DMPE liposomes (Kolber and Haynes, 1979; Pryor et al., 1983). In addition, both CHEMS and TS alone were able to form liposomes with encapsulation volumes of 1.0 and 2.2 µl per µmole lipid respectively.

4.2. Thermotropic Behavior of DPPC-CHEMS and DPPC-TS Mixtures

The partitioning of CHEMS and TS into phospholipid bilayers was studied by differential scanning calorimetry. For these studies DPPC and DMPE were used as model phospholipids. The thermograms of DPPC-CHEMS mixtures are shown in Fig. II-1. The pretransition of DPPC was not completely abolished by the addition of 5 mole% cholesteryl hemisuccinate (Fig. II-1), rather it was shifted to a lower temperature as a shoulder in the endothermic peak. The shape of the main transition of DPPC was not changed by the addi-

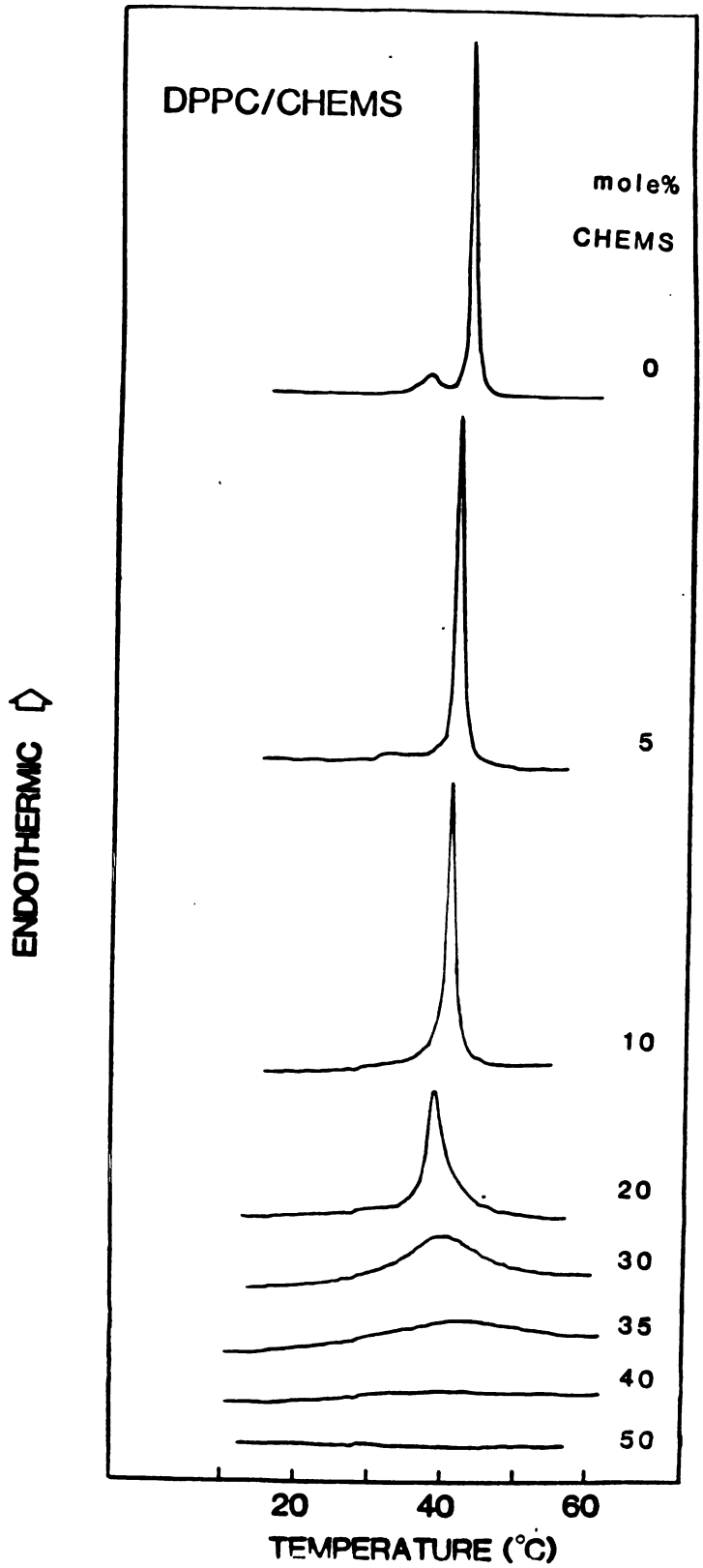


Figure II-1. Differential scanning calorimetry thermograms of DPPC-CHEMS mixtures with 0, 5, 10, 20, 30, 35, 40, 50 mole% of CHEMS.

tion of CHEMS up to 10 mole% (Fig. II-1). The addition of 20 mole% CHEMS broadened the half height width of the excess heat curve of DPPC (Fig. II-1), while the curve shape became asymmetric with a sharp transition at the lower temperature and a shoulder at the higher temperature. The peak of the transition curve shifted continuously to lower temperature with the addition of CHEMS until 20 mole% (Fig. II-1,II-2). The sharp transition disappeared with the addition of 30 mole% CHEMS, the transition was further broadened and the peak transition temperature shifted to a higher temperature (Fig. II-1, II-2). The transition curve of DPPC-CHEMS at 65:35 molar ratio was further broadened (Fig. II-1). The increase in total transition range of DPPC by the addition of CHEMS is seen clearly in Fig. II-2, both the onset and the end of the transition are shifted away from the maximum of the transition. The phase transition of DPPC was barely detectable with 40 mole% CHEMS and was completely abolished at a 1:1 molar ratio of DPPC:CHEMS (Fig. II-1). The suppression of transition is best demonstrated by the decrease in the transition enthalpy proportional to the content of CHEMS, and its reduction to zero between 40 and 50 mole% CHEMS (Fig. II-3, circle).

DPPC/TS mixtures displayed a distinctive thermotropic behavior significantly different from DPPC/CHEMS mixtures. The incorporation of 5 mole% TS suppressed the pretransition, broadened the main transition endotherm, and decreased

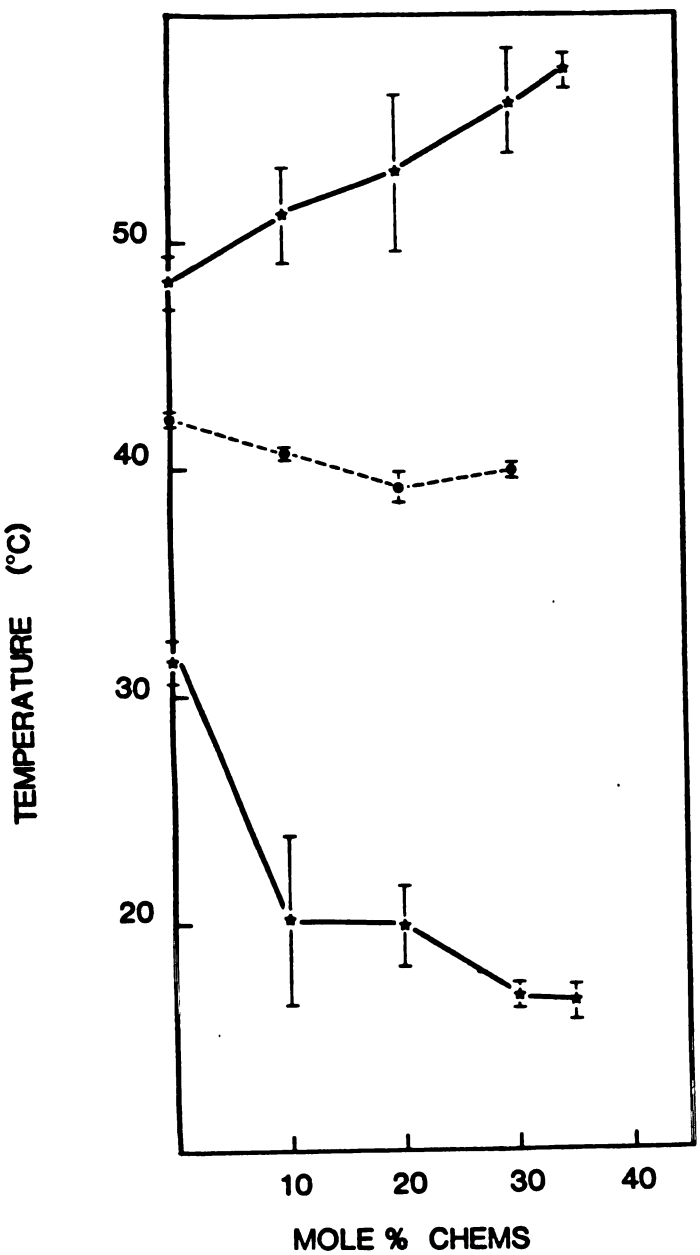


Figure II-2. Partial phase diagram of DPPC-CHEMS mixtures. ★, onset and end of the heating curve. ●, the maxium of the endothermic excess heat curve. Each point is the average value of four different preparations. Error bar is the standard deviation.

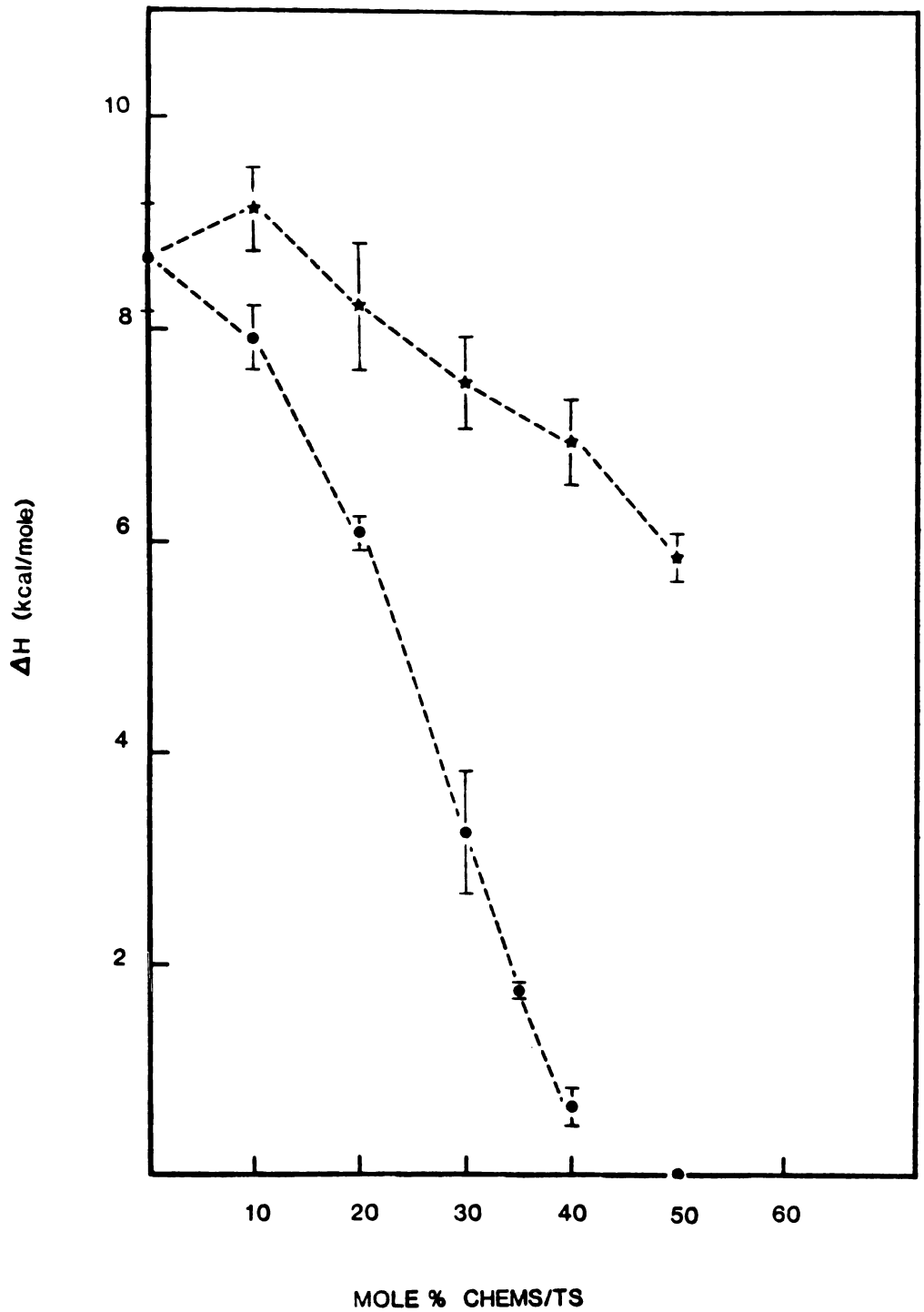


Figure II-3. Plot of the total transition enthalpy(kcal per mole of DPPC) against the mole fraction of CHEMS(●) and TS(★).

the peak temperature of DPPC (Fig. II-4). A further increase in the total transition range of DPPC was observed with 10 and 20 mole% of TS. Incorporation of TS beyond 30 mole% showed little effect on the peak width of the excess heat curve (Fig. II-4). This is clearly demonstrated in the partial phase diagram of DPPC-TS (Fig. II-5). The transition curve remained asymmetric with a broad shoulder toward lower temperatures. With additional TS, the endotherm moved to lower temperatures (Fig. II-4 & II-5). Only partial scans were performed on the mixtures containing more than 60 mole% TS, since the onset of the transition was lower than the temperature(about $-10^{o}C$) that aqueous samples can be studied in the DSC without cryoprotectants. In our studies no cryoprotective agents were added to extend the thermoscan to lower temperatures. The temperature at the maximum of the endothermic curve at 80 mole% TS is about $30^{o}C$ lower than that of DPPC (Fig. II-4). The phase transition of DPPC is still detectable at 90 mole% TS. Thus even at a ratio of 9 to 1, TS is not able to completely abolish the main transition of DPPC. The plot of enthalpy against mole% TS added (Fig. II-3) shows only a relatively small decrease in transition enthalpy with the incorporation of d-tocopherol succinate. It should be noted that with TS alone no phase transition can be detected by DSC between $-22^{o}C$ and $62^{o}C$.

4.3. Thermotropic Behavior of DMPE-CHEMS and DMPE-TS Mixtures

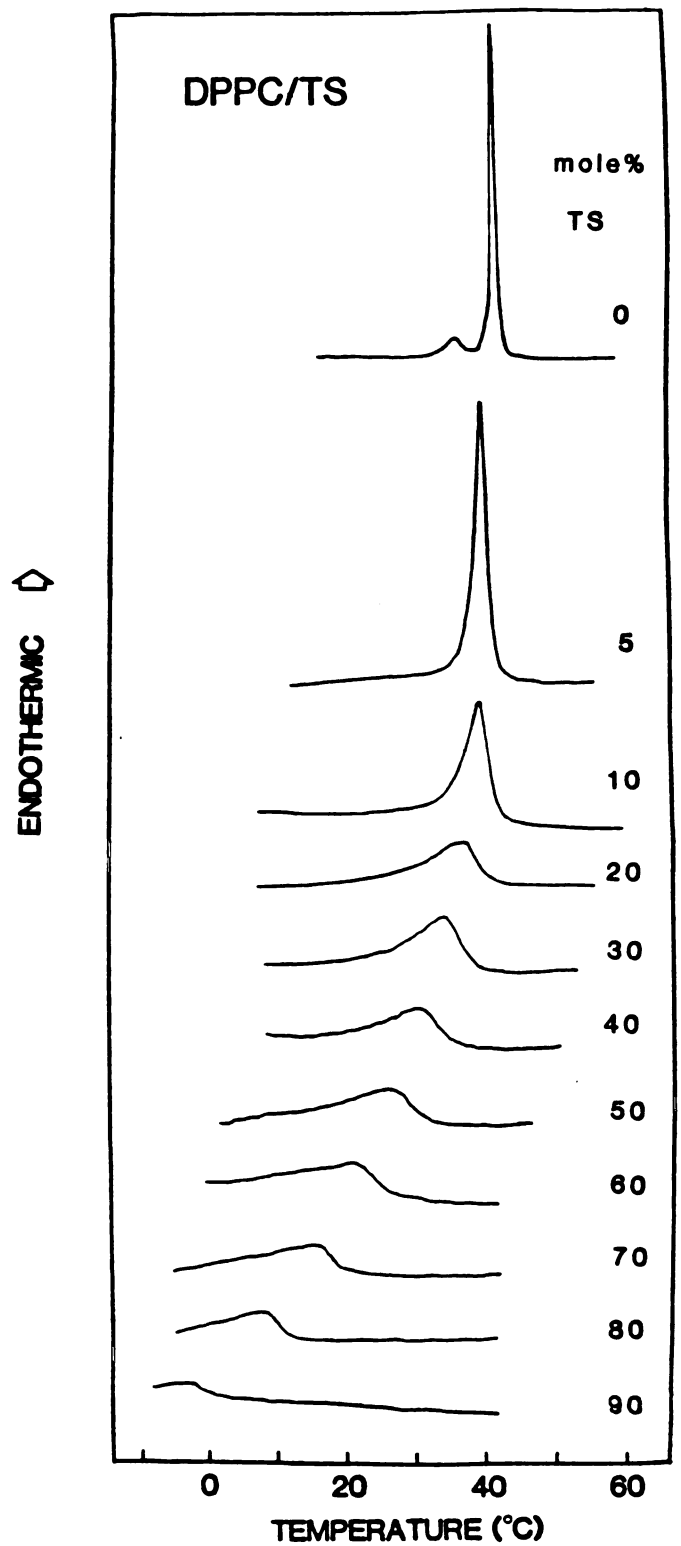


Figure II-4. Thermograms of DPPC-TS mixtures with 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 mole% of TS.

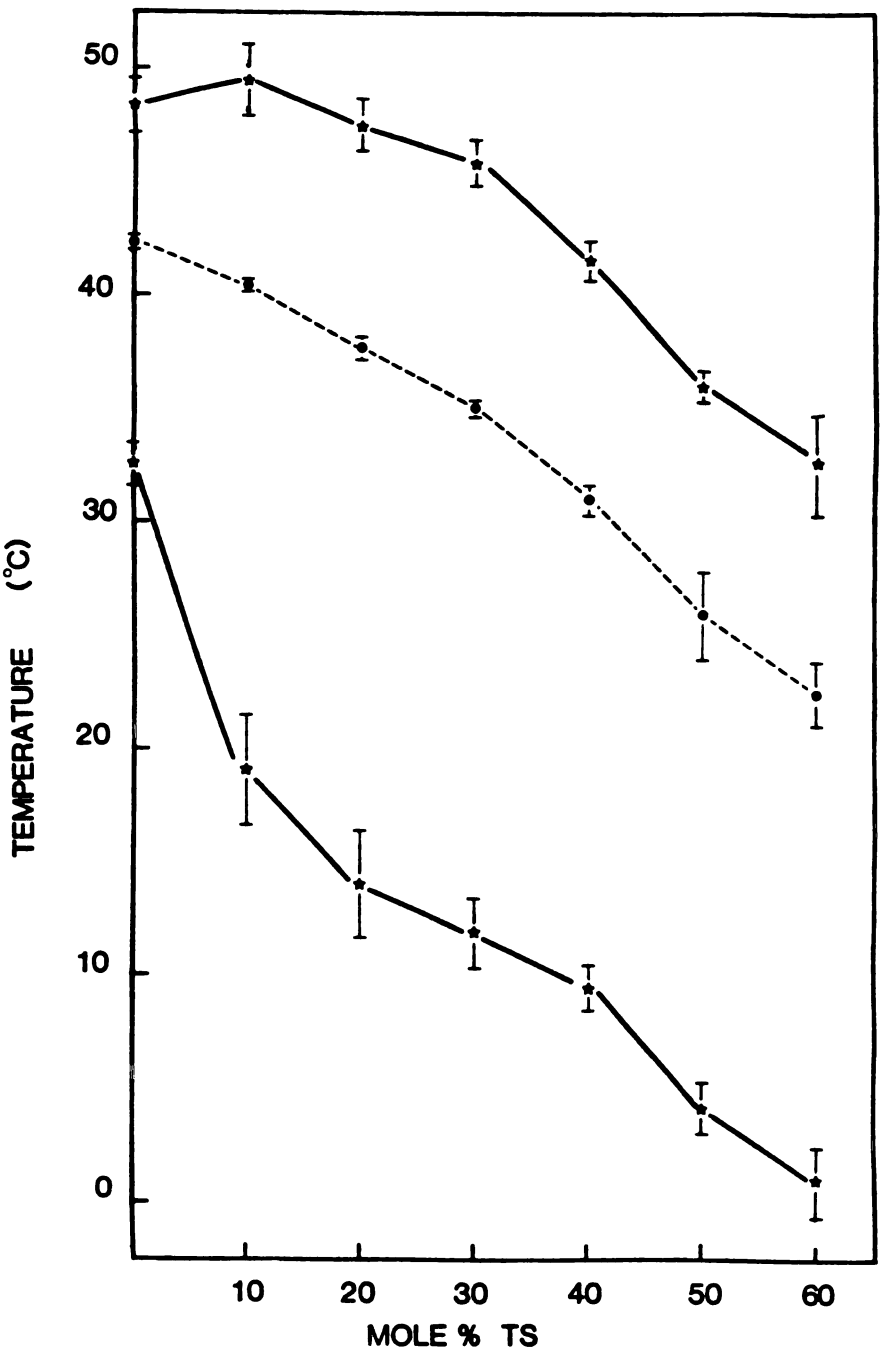


Figure II-5. Partial phase diagram of DPPC-TS mixtures. ★, the beginning and the end of the phase transition. ●, the maximum of the excess heat curve. Each point represents the average value of four different samples.

To determine if the headgroup had a significant influence on the behavior of these compounds in membranes, the interactions between CHEMS or TS and DMPE were also studied by DSC. The addition of 5 mole% CHEMS to DMPE significantly increased the peak width of the excess heat curve (Fig. II-6). 10 Mole% CHEMS further broadened the endothermic curve, while at 20 mole% CHEMS the sharp transition disappeared. The addition of more CHEMS to DMPE led to further broadening of the transition curve (Fig. II-6). In addition to the increase in the temperature range of the transition, the maximum of the endothermic curve decreased continuously with the increase in molar ratio of CHEMS (Fig. II-7). At 50 mole% CHEMS the transition was no longer detectable (Fig. II-6).

DMPE-TS mixtures displayed thermotropic behavior similar to DPPC-TS. Upon incorporation of 10 mole% TS, the excess heat curve of DMPE was broadened and became highly asymmetric with the peak maximum at the high temperature end (Fig. II-8). Occasionally double peaks were observed in the thermograms of samples containing 10 to 20 mole% of TS, which suggests that phase separation during sample preparation might have been occurring. Little change in the endothermic peak width was observed with the incorporation of more than 20 mole% TS in DMPE (Fig. II-8 & II-9). The transition curve was shifted toward lower temperatures as additional TS was incorporated (Fig. II-8 & II-9). The

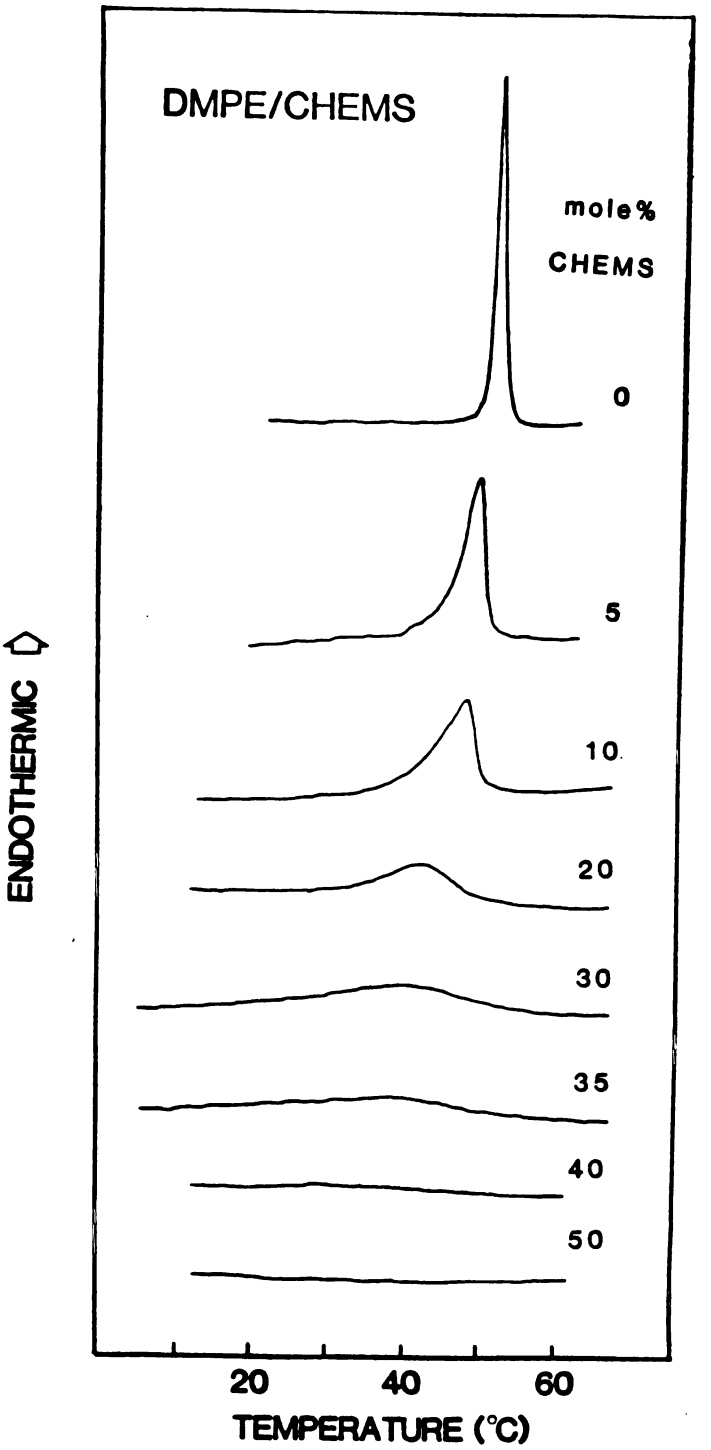


Figure II-6. Thermograms of DMPE-CHEMS mixtures with 0, 5, 10, 20, 30, 35, 40, 50 mole% of CHEMS.

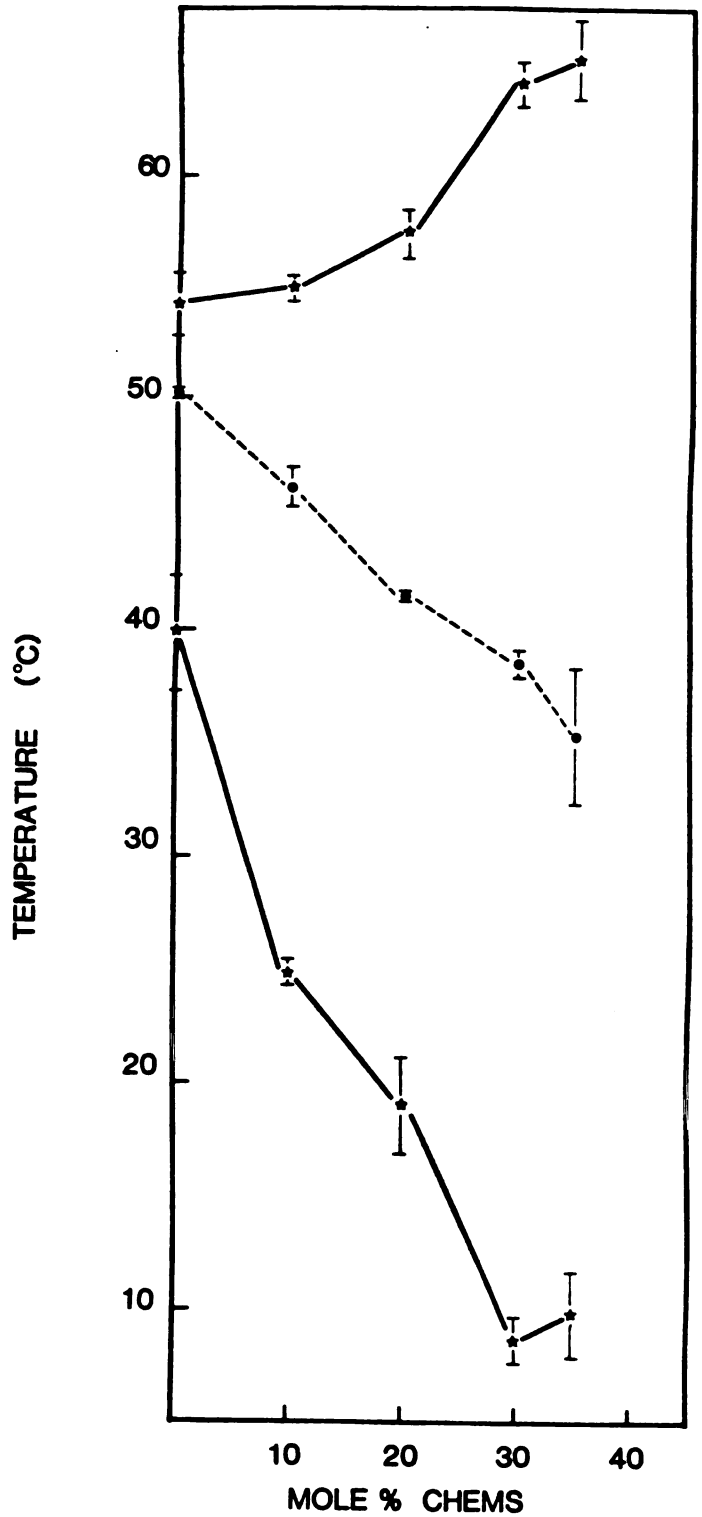


Figure II-7. Partial phase diagram of DMPE-CHEMS mixtures. ★, the onset and end of the gel-liquid cryatalline phase transition deduced from the heating curve. ●, the maximum of the endothermic excess heat curves. The points are the average of three different preparations for each composition.

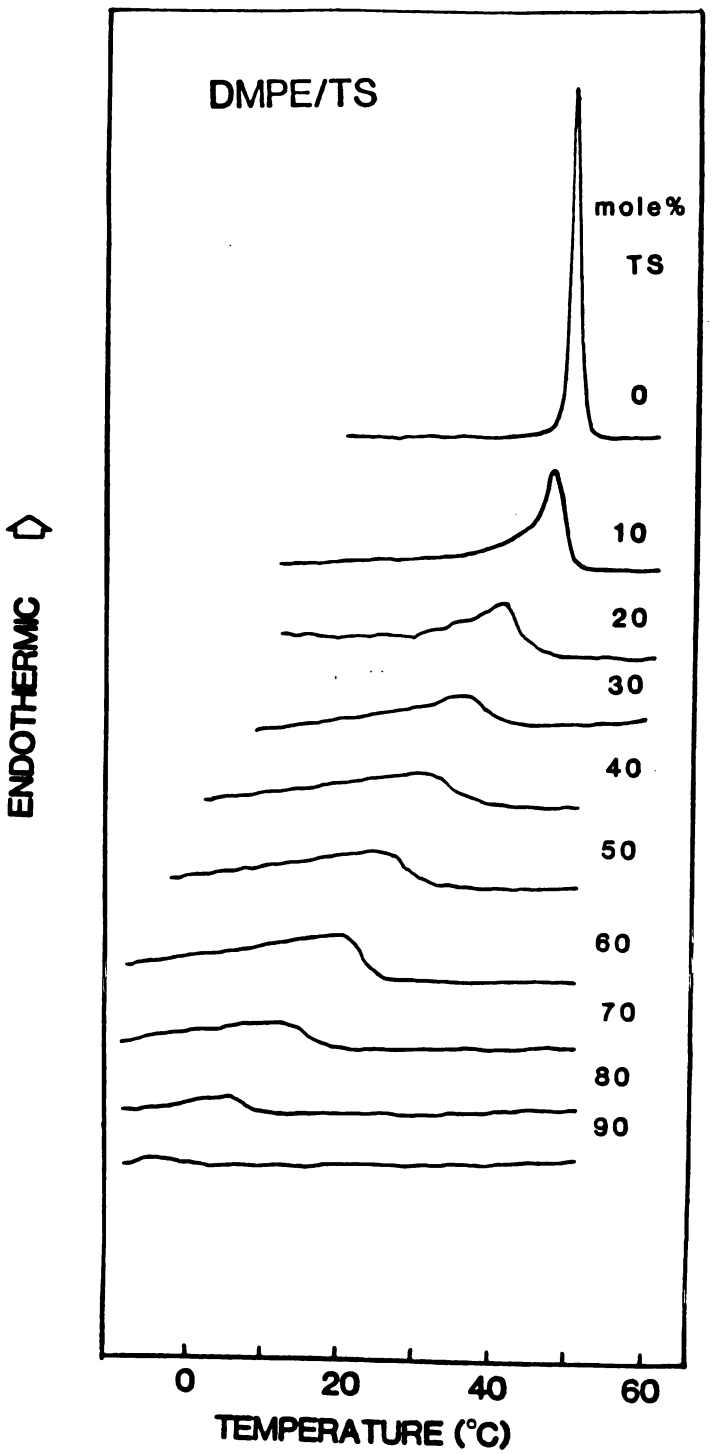


Figure II-8. Thermograms of DMPE-TS mixtures with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 mole% of TS.

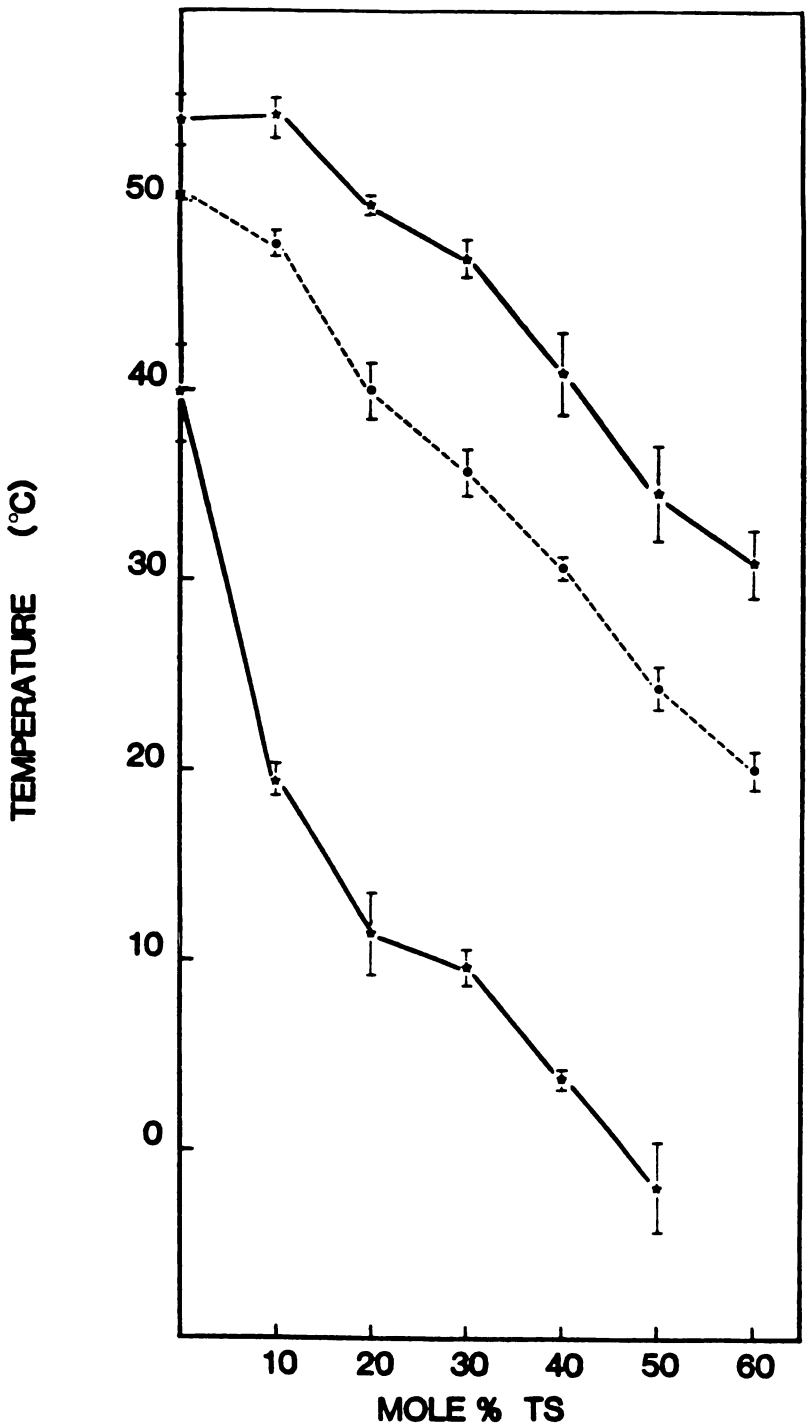


Figure II-9. Partial phase diagram of DMPE-TS mixtures. ★ and ● are the same as defined in Fig. II-2. Each point represent the average value of 3 different samples.

shift in the peak temperature of the endothermic curve was greater for DMPE-TS than for DPPC-TS. At 80 mole% TS the temperature at the maximum of the transition curve was about 40°C lower than that of DMPE (Fig. II-8). Similar to the DPPC-TS mixture, the phase transition of DMPE/TS at 1/9 molar ratio was still visible (Fig. II-8). However, the transition enthalpy of DMPE decreased only slightly with the addition of TS, whereas a plot of the transition enthalpy vs mole ratio CHEMS in DMPE exhibited an almost linear decrease (Fig. II-10). The intercept of the extrapolated straight line at the abscissa(zero enthalpy) is around 42 mole% CHEMS.

5. Discussion

5.1. Succinate Esters of Cholesterol and Tocopherol

Both cholesterol and α-tocopherol have limited solubility in phospholipid bilayers. Cholesterol can be incorporated into phospholipid membranes up to 50 mole%, beyond this limit cholesterol is separated from phospholipid bilayers (Ladbrooke et al., 1968; Lecuyer and Dervichian, 1969). Although the solubility of tocopherol in phospholipid membranes has not been established, lecithin liposomes containing more than 20 mole% tocopherol are difficult to prepare (Bellemare and Fragata, 1981). The attachment of a charged ester greatly enhances the partitioning of

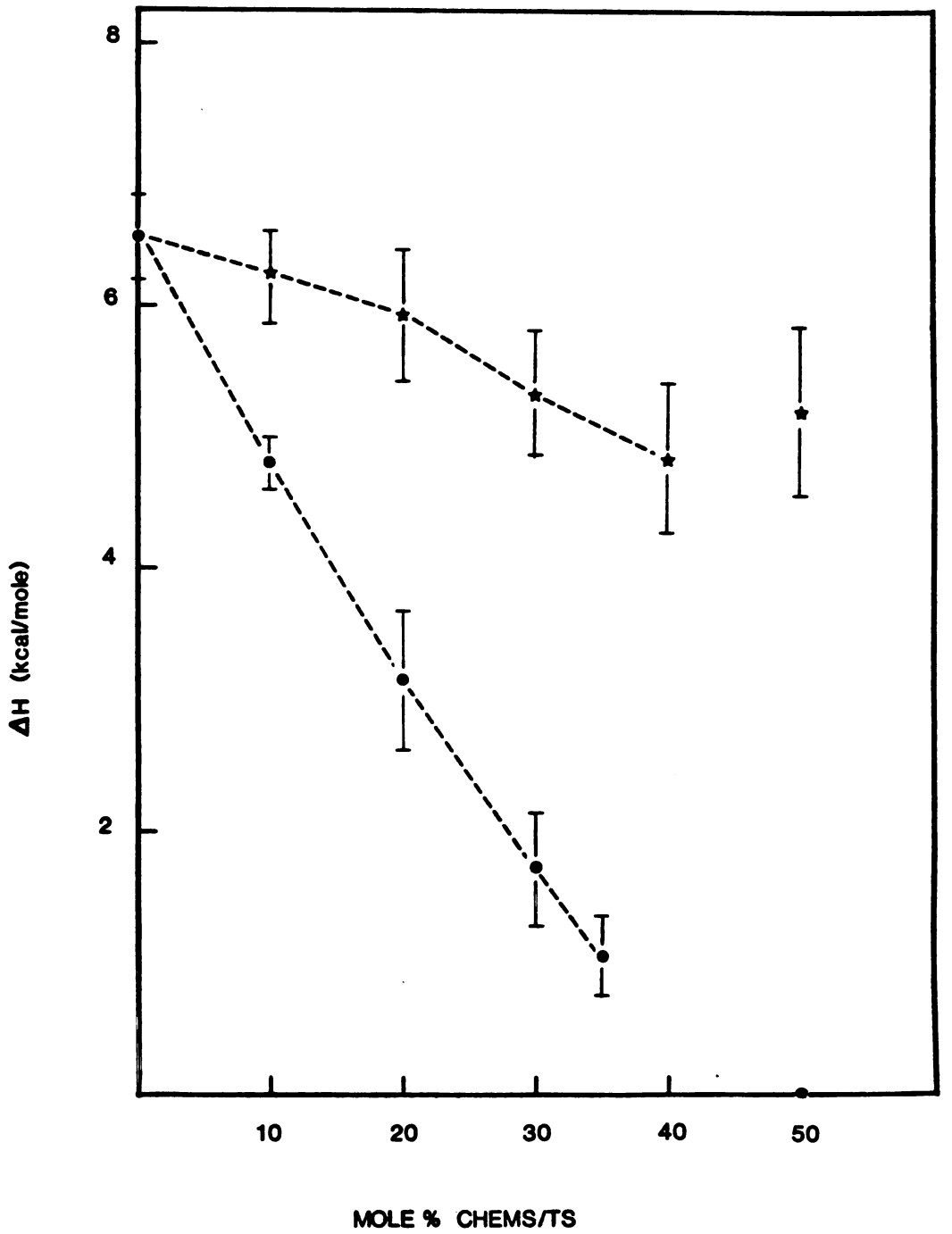


Figure II-10. Plot of the total transition enthalpy(kcal per mole of DMPE) vs. mole fraction of CHEMS(●) and TS(★).

cholesterol and tocopherol into phospholipid bilayers. Both CHEMS and TS can be dissolved in phospholipid membranes far beyond the limits of the parent compounds. The increased incorporation of CHEMS and TS does not affect the integrity of the phospholipid bilayers, as indicated by ^{3}H-sucrose encapsulation. Furthermore, the enhanced hydrophilicity of CHEMS and TS helps stabilize DMPE in bilayer membranes. In contrast to the instability of DMPE lipid vesicles at neutral pH (Stolley and Vail, 1977; Kolber and Haynes, 1979; Pryor et al., 1983), liposomes of DMPE can be prepared with 20 mole% CHEMS or TS. The ability to stabilize PE in bilayers is a property CHEMS and TS have in common with other amphiphiles such as phosphatidylcholine (Kolber and Haynes, 1979).

In addition to enhanced solubility in phospholipid membranes, CHEMS and TS are able to form multilamellar vesicles by themselves. 1 Hour sonication of these liposomes (at 20μmole/ml) resulted in small unilamellar vesicles (electron micrography not shown). The ease in the preparation of CHEMS vesicles is in contrast to other charged cholesterol esters such as cholesteryl phosphorylcholine, cholesteryl sulfate and cholesteryl poly(ethyleneglycol). For these cholesterol esters, the addition of equimolar cholesterol is required for the formation of unilamellar vesicles (Brockerhoff and Ramsammy, 1982). The difference between CHEMS and these charged esters is likely due to the size differ-

ence in the charged headgroups (Brockerhoff and Ramsammy, 1982).

5.2. Role of the Hydroxyl Group in Cholesterol-Phospholipid Interactions

The widespread occurrence of cholesterol in mammalian cell membranes has been the impetus for a large number of physico-chemical studies on the interaction of cholesterol with model phospholipid membranes (reviewed in Demel and de Kruijff, 1976; Presti et al., 1982). These studies have provided an understanding of many features of cholesterol-phospholipid interaction. However, the role of 3β-OH group of the sterol remains controversial. It has been shown that neither the 3α-OH derivative, the 3-keto derivative, nor the 3-thiol derivative of cholesterol can mediate the membrane condensing effect (Hsia et al., 1972; Parkes et el., 1982), or decrease the permeability to glycerol and erythritol (Demel et al., 1972). These studies have led to the suggestion that the 3β OH group of cholesterol forms a hydrogen bond with the carbonyl group (Brockerhoff, 1974; Huang, 1976, 1977) or with the glycerol oxygen (Presti et al., 1982) of the phospholipid. However studies utilizing a variety of both physical techniques (Yeagle et al., 1975; Clejan et al., 1979; Bush et al., 1980) and chemical derivatives of phospholipids (de Kruijff et al., 1973; Fong et al., 1977; Tirri et al., 1977; Clejan et al., 1979; Bartholow and Geyer, 1982) have indicated that there is no direct

hydrogen bond between the sterol and the phospholipid. An alternative approach is to work with derivatives of cholesterol with a modification at the 3β OH position. We have used the succinate ester of cholesterol modified at the 3β OH position as a model cholesterol compound and examined its effect on the thermotropic properties of phospholipid membranes using differential scanning calorimetry. The thermotropic behavior of the CHEMS mixtures closely resembles that of DPPC-cholesterol (Ladbrooke et al, 1968; Mabrey et al, 1978; Estep et al., 1978). Although the calorimeter used here is of lower sensitivity, many features of the DPPC-cholesterol mixtures revealed in high sensitivity DSC can be seen in the present studies. For example, the half-height width of the endothermic curve of DPPC is not changed with 10 mole% of CHEMS (Fig. II-1) or cholesterol (Estep et al., 1978). The broadening of the transition peak was observed at 20 mole% for both CHEMS and cholesterol. Furthermore like cholesterol, the endothermic peak of DPPC-CHEMS(80:20) is asymmetric. With the increase of CHEMS from 20 mole% to 30 mole%, the sharp transition at 38.5°C disappeared and the shoulder at 40°C became the maximum of a broad transition curve (Fig. II-1). The sharp transition observed with mixtures containing 20 mole% CHEMS(or less) has been interpreted to be due to pure DPPC domains (Mabrey et al., 1978, Estep et al., 1978, Presti et al., 1982). The abrupt broadening of the transition of DPPC as CHEMS is increased from 20 mole% to 30 mole% supports the model of a

2:1 phospholipid/sterol complex originally proposed by Lecuyer and Dervichian (1969) and emphasized recently by Presti et al. (1982). Further addition of CHEMS or cholesterol broadens the transition curve until the gel-liquid crystalline transition is completely suppressed. With CHEMS, the complete disappearance of the main transition occurs at a mole ratio between 40-50% CHEMS (Fig. II-3). Thus CHEMS, within experimental error, is as effective in complexing with DPPC as cholesterol, judging from the concentration of CHEMS required to abolish the sharp transition and to suppress the total transition.

The effects of CHEMS on the thermotropic behavior of a saturated phosphatidylethanolamine, DMPE, are also analogous to those of cholesterol (van Dijck et al., 1976; Blume, 1980). The addition of 5 mole% CHEMS (Fig. II-6) or cholesterol (Blume, 1980) broadens the endothermic peak of DMPE. Moreover a shoulder is observed at the lower temperature region of the transition in DMPE mixtures containing a low molar ratio of CHEMS (Fig. II-6) or cholesterol (van Dijck et al., 1976; Blume, 1980). The phosphatidylethanolamines have a higher transition temperature than the comparable phosphatidylcholine, an effect ascribed to an intermolecular hydrogen bond between adjacent PE molecules (Hitchcock et al., 1974; Chapman, 1975; Boggs, 1980). The incorporation of cholesterol into a PE membrane causes a downshift of the transition temperature (van Dijck et al.,

1976; Blume, 1980). This is believed to be due to the intercalation of the cholesterol between PE molecules resulting in a disruption of the hydrogen bonding network (Blume, 1980; Blume and Griffin, 1982). The extent of the lowering of the transition temperature (Fig. II-7) and the almost linear decrease of the transition enthalpy (Fig. II-10) with increasing amount of CHEMS are, within experimental error, quantitatively the same as that observed with cholesterol (van Dijck et al., 1976; Blume, 1980). Moreover the significant broadening of the transition at around 20 mole% CHEMS is the same as as that observed with cholesterol (van Dijck et al., 1976; Blume, 1980). This implies that CHEMS may also form a 1:2 complex with DMPE.

The results here clearly demonstrate that CHEMS can form a complex with phospholipid as effectively as cholesterol. Shinitzky et al. (1979) have demonstrated that CHEMS increases the microviscosity of mammalian cell membranes as measured by diphenylhexatriene fluorescence polarization. More recently Simmonds et al. (1984) showed the identical membrane ordering effects of CHEMS and cholesterol. The succinate esterification of 3β-OH group, therefore, does not affect the interaction between cholesterol and phospholipids.

Recently the biological activity of a number of charged cholesterol esters has also been studied. Lyte and Shinitzky (1979) measured the effect of cholesteryl phosphoryl-

choline on the microviscosity of lipid vesicles composed primarily of phosphatidylcholine. They found the change to be similar to that caused by the same molar ratio of cholesterol. Colombat et al. (1981) arrived at a similar conclusion for the effect of cholesterylphosphate. Although the increase in the microviscosity cannot be related to the ability of these derivatives to form a sterol-phospholipid complex, these studies suggest that replacing the 3β-OH group with a hydrophilic ester does not affect the membrane ordering activity of cholesterol. More recently Demel et al. (1984) examined the interaction of a number of non-ionic ether derivatives of cholesterol with phospholipids. They found that cholesteryl (2-hydroxyl)-3-ethyl ether is as effective as cholesterol in the membrane condensing effect, the reduction of glucose permeability, and the suppression of the gel-fluid transition of phospholipid. Demel et al. (1984) concluded that the 3β hydroxyl group is not necessary for the sterol effect to occur. Instead they proposed that the orientation of the sterol and the presence of oxygen moiety at the interface are important for the sterol-phospholipid interaction; the latter may involve hydrogen bonding between the sterol headgroup and the bound water system.

These previous results with other cholesterol derivatives are complemented by our finding that the attachment of a succinate group to the 3β OH position of cholesterol does

not significantly change the interaction with the two phospholipids studied here. Thus it is unlikely that sterol effects involve a specific interaction between the 3β OH of cholesterol and the phospholipid. The role of the 3β-OH group seems to be positioning the sterol ring near the aqueous interface so that the ring structure can maximize the van der Waals interactions with the acyl chains of the phospholipid, an argument that Cadenhead and Muller-Landau (1979) have previously postulated. The reduced ability of epicholesterol and 3-keto cholesterol in forming a complex with phospholipid can be explained by the geometry of the α-OH and keto group, which position the sterol in an orientation not favorable for van der Waals interactions with the phospholipid (Cadenhead and Muller-Landau, 1979). A free 3β-OH group, however, is not necessary for the sterol-phospholipid interaction. Our results and those by Demel et al. (1984) suggest that the correct alignment of the sterol in phospholipid bilayers can be maintained if the 3β-OH group is substituted with a hydrophilic ester (such as succinate), or with a ether and additional 2-OH groups.

5.3. Role of the Hydroxyl Group in Tocopherol-Phospholipid Interactions

Tocopherol is a well known biological antioxidant that has been proposed to have a structural function in membranes containing polyunsaturated fatty acyl groups (Diplock and Lucy, 1973). Despite its biological importance, surpris-

ingly little is known about its behavior in model membranes. Its effect on the permeability of the bilayers is complex and depends on the acyl chain composition of the phospholipids and molar ratio of tocopherol in the bilayer. In egg phosphatidylcholine vesicles the addition of d-tocopherol at less than 20 mole% slightly decreases the permeability to small molecules such as glucose and chromate (Diplock et al., 1977; Fukuzawa et al., 1979; Stillwell and Bryant, 1983). However at 25 mole% in egg phosphatidylcholine vesicles Pr^{3+} permeability increases 48 fold (Cushley and Forrest, 1977). In DPPC liposomes the addition of 16 mole% tocopherol increases the permeability to ascorbate 3 fold (Srivastava et al., 1983). When added to lipid vesicles composed of saturated phospholipids, tocopherol slightly lowers the transition temperature (1-2^{o}C) and significantly broadens the phase transition as observed by DSC; the transition is abolished at 25 mole% tocopherol (Massey et al., 1982). Broadening of the phase transition has also been observed by fluorescence polarization (Fukuzawa et al., 1980) and by ESR using the partitioning of the spin label tetramethylpiperidine-N-oxyl into bilayers containing tocopherol (Srivastava et al., 1983).

Using the succinate derivative of tocopherol, we can explore the role of the hydroxyl group in the tocopherol-phospholipid interaction. We have found that the interaction of TS with both DPPC and DMPE is substantially dif-

ferent from that of CHEMS (Figure II-1,4,6,8) or tocopherol (Massey et al., 1982). At 5 mole%, TS abolishes the pre-transition of DPPC and broadens the peak width significantly, similar to the effect of tocopherol at this mole % (Massey et al., 1982). At 20 mole% TS the peak width of both DPPC and DMPE is further increased. Above this ratio there is little change in the endothermic curve shape but the transition peak continues to shift to lower temperatures, a finding that is considerably different from that reported for tocopherol (Massey et al., 1982). This absence of an effect on the transition of the host lipid (DPPC or DMPE) can be clearly observed from the enthalpy of the transition for the two systems (Figure II-3, II-10), which decreases only 2 Kcal/mole for DPPC and 1 Kcal/mole for DMPE at 50 mole percent TS. Due to the small decrease in the transition enthalpy of DPPC by the addition of TS, the gel-liquid crystalline transition remains detectable with 90 mole% TS. This clearly shows that there are DPPC or DMPE-rich domains in which a cooperative phase transition still occurs. The lowering of the transition temperature with increasing TS in the bilayer may be explained by an impurity defect surrounding the phospholipid domains (Lee, 1977a).

Massey et al. (1982) had reported that tocopherol acetate at 25 mole% behaved almost exactly like tocopherol in abolishing the transition of DMPC. However Schmidt et al. (1976) had not observed such an effect of tocopherol acetate

in DPPC bilayers. Rather they observed a broadening of the transition and a decrease in the transition temperature up to 40 mole% tocopherol acetate at which point the ester formed a separate phase. A similar result has been reported by Srivastava et al. (1983). They found that tocopherol acetate at 16 mole% depressed the transition temperature of DPPC to 28°C while the sharpness of the transition was retained. By comparing the effects of tocopherol and tocopherol acetate on the phase transition of DPPC and on the ^{13}C relaxation time and line width of the two compounds, Srivastava et al. (1983) proposed that the hydroxyl group is essential for the interaction of tocopherol with phospholipids. However their results do not differentiate between the disruption of a tocopherol-phospholipid interaction via a hydrogen bond, and the displacement of tocopherol from the aqueous interface into the interior of the bilayer due to the increased hydrophobicity of the tocopherol acetate. The DSC results reported here for TS, which will be anchored at the aqueous interface, support the suggestion that the hydroxyl group of tocopherol is of major importance to its ability to interact with phospholipids. The anchoring of tocopherol at the interface of membranes would position it advantageously to exert its antioxidant effect.

6. Conclusion

The use of the succinate analogues of cholesterol and tocopherol allows an appraisal of the role of the respective hydroxyl groups in their interactions with phospholipids. In the case of cholesterol the 3β-OH appears to be sufficient, but not necessary, for the condensing effect of cholesterol to occur. The exact requirement at the 3 position of cholesterol for the condensing effect seems to be a hydrophilic group that permits the sterol ring to be positioned at the interface so that the van der Waals interactions with the acyl chains of the phospholipids can be maximized. This must be contrasted to tocopherol where the hydroxyl group is necessary to maximize the interaction with phospholipids. Although a hydrogen bond to the adjacent phospholipid is an attractive possibility the exact nature of the tocopherol-phospholipid interaction must still be determined.

CHAPTER III

ACID AND CALCIUM INDUCED STRUCTURAL CHANGES IN PHOSPHATIDYLETHANOLAMINE MEMBRANES STABILIZED BY CHOLESTERYLHEMISUCCINATE

1. Summary

The membrane stabilization effect of cholesterylhemisuccinate (CHEMS) and sensitivity of the CHEMS-phosphatidylethanolamine membranes to protons and calcium ions were studied by differential scanning calorimetry, freeze-fracture electron microscopy and ^{31}P-NMR. (1) At neutral pH, the addition of 8 mole% CHEMS to transesterified egg phosphatidylethanolamine (TPE) raised the lamellar-hexagonal transition temperature of TPE by 11°C. Stable bilayer vesicles were formed when the incorporated CHEMS exceeded 20 mole%. (2) At a pH below 5.5, the protonation of CHEMS enhanced the formation of the hexagonal phase (H_{II}) of TPE. At 25 mole% CHEMS the bilayer-hexagonal transition temperature was lowered by 30°C at pH 4.5. (3) The endothermic acid-induced hexagonal transition of TPE-CHEMS was suppressed at 35 mole% CHEMS. However, ^{31}P-NMR and electron microscopy indicated that a lamellar-hexagonal transition still occurred at this composition. (4) The main transition of TPE was not affected by the protonation of the

incorporated CHEMS, indicating that no macroscopic phase separation occurred in TPE/CHEMS mixtures at low pH. (5) In contrast to the H_{II}-promoting effect of H^+, the neutralization of the negative charge on TPE/CHEMS by Ca^{++} resulted in aggregates which remained in the lamellar structure even at the hexagonal transition temperature of TPE. It is suggested that calcium might form a complex between CHEMS in apposed bilayers.

2. Introduction

Negatively charged phospholipids such as phosphatidylserine and phosphatidylglycerol have been implicated in important biological functions of biomembranes because the structure and properties of these anionic phospholipids are charge dependent. The order-fluid transition of acidic phospholipids can be regulated by changes in pH or divalent ion concentration (Trauble and Eibl, 1974; Trauble, 1976). Anionic phospholipids neutralized with protons or calcium are highly ordered (Newton et al., 1978; Harlos and Eibl, 1980a; Liao and Prestegard, 1981) and are segregated from other membrane lipids, resulting in the destabilization of the membranes (Jacobson and Papahadjopoulos, 1975; Galla and Sackmann, 1975; Tokutomi et al., 1980). The interaction of protons, divalent or polyvalent cations with acidic phospholipids also leads to membrane fusion (Papahadjopoulos et

al., 1974; 1976; 1977).

While anionic phospholipids have been subjected to extensive studies, other acidic lipids have received little attention. Recent work in this and other laboratories have demonstrated interesting properties of cholesteryl phosphate (Colombat et al., 1981) and cholesterylhemisuccinate (Ellens et al., 1984a; Chapter II). Despite the lack of 3β-OH group these cholesterol esters retain the properties and function of cholesterol (Colombat et al., 1981; Chapter II). Among other anionic cholesterol esters, cholesteryl sulfate is probably an important example, cholesteryl sulfate has been found in brain, adrenal, aorta, liver, kidney and plasma (Moser et al., 1966), and is located in the intestinal brush border membrane in abundant amounts (Pascher and Sundell, 1977). In human erythrocyte membranes, cholesteryl sulfate has been shown to be protective against hypotonic hemolysis (Bleau et al., 1974). Based on the specific uptake of cholesteryl sulfate by spermatoza, Langlais et al.(1981) hypothesized the role of cholesteryl sulfate in membrane stabilization and enzyme inhibition during sperm storage within the epididymis, and the cleavage of sulfate moiety leading to sperm capacitation and fertilization. The pathological state in patients with recessive X-linked ichthyosis has been suggested to be related to the increased content of cholesteryl sulfate in the stratum corneum, resulting from a cholesterol sulfatase deficiency in the epidermis (Williams

and Elias, 1981). Thus negatively charged cholesterol esters are probably playing important roles, similar to those of acidic phospholipids, in the structure and functions of biomembranes.

In Chapter II we have used cholesterylhemisuccinate as a model compound to study the interaction between phospholipids and charged cholesterol esters. In the studies described in this Chapter, we have also used CHEMS to demonstrate the membrane stabilization effect of anionic cholesterol esters; and to study pH and Ca^{++} induced structural changes in membranes containing CHEMS. We have found that protons and calcium have different effects on the phase behavior of CHEMS-PE liposomes. The response of PE-CHEMS membranes to protons and calcium ions may be related to the biological functions of acidic cholesterol esters.

3. Materials and Methods

3.1. Lipids and Chemicals

Egg phosphatidylcholine (EPC), transesterified egg phosphatidylethanolamine (TPE), and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterylhemisuccinate was purchased from Sigma. All lipids produced a single spot in silica gel thin layer chromatography with chloroform/methanol/acetic acid/water (100/50/14/16) and

chloroform/methanol/ammonia/water (115/45/2/6), and were stored under nitrogen at -50°C. D_2O was obtained from Aldrich. All other chemicals were of reagent grade or better.

3.2. Aqueous Dispersions of Lipids

Lipids were first deposited onto a round bottom flask by removing the organic solvent through rotatory evaporation. Lipids were then hydrated in Tris-NaCl buffer(50mM Tris, 110mM NaCl, pH 7.4) under positive nitrogen pressure. Brief sonication(2-5 mins) was applied to lipids that did not easily hydrate. The pH was adjusted by dialyzing against buffer (50mM acetate, 110mM NaCl, pH 3.5-5.5), or by direct mixing with a large volume (>10X) of concentrated acidic buffer (150mM acetate, 25mM NaCl). The final pH of the dispersions was checked with two sets of Hydrion Microfine pH test paper (Micro Essential Lab., N.Y.) with overlapping pH ranges. The pH for all samples was measured at 25°C. The Ca^{++} concentration was altered by dialyzing against Tris-NaCl buffer containing calcium. To minimize the oxidation of phospholipids, samples were freshly prepared for each study and dialysis was performed with argon bubbling through the buffer.

3.3. Differential Scanning Calorimetry

Samples for DSC were concentrated by centrifugation in an Eppendorf centrifuge(12,800g, 1 min) and the pellet was dispersed in 50µl of the same buffer used for sample

preparaiton. 17µl of the final dispersion was sealed in an aluminum sample pan. The lipids precipitated by acid and calcium ion were difficult to redisperse in buffer, and were applied directly onto the sample pan followed by the appropriate amount of acidic or Ca^{++}-containing buffer. DSC measurements were made with a Perkin-Elmer DSC-2 calorimeter operating at a sensitivity of 1 mcal/sec and a scanning rate of $5^{o}C$/min. Continuous heating(2) and cooling(1) runs were performed on each sample. At least two samples were studied for each composition of lipid mixtures. Unless specified, no cryoprotectants were added to the sample and each scan started at $265^{o}K$. Because the phase transition of phospholipids of natural origin is broad, the maximum of the excess heat curve was taken as the transition temperature.

3.4. Phosphorous-31 Nuclear Magnetic Resonance

Lipid samples for ^{31}P-NMR were deposited on the vessel by removing the chloroform in a rotatory evaporator at reduced pressure followed by an additional hour under high vacuum, and then hydrated in Tris-NaCl buffer containing 10% D_2O at a final concentration of 40-50 µmole of phospholipid per ml. For samples where additional dialysis was required for the adjustment of the pH or Ca^{++} concentration, D_2O was added after dialysis. ^{31}P-NMR spectra were recorded on a Varian XL-100 Spectrometer operating in Fourier transform mode at 40.5 MHz. Samples were placed in 12mm tubes and equilibrated at the temperature to be studied for at least

10 mins before signal acquisition. Accumulation free induction decays were obtained from 3,000-6,000 transients with a 45° pulse angle, 0.5 sec interpulse time and a delay time of 200 µsec. A sweep width of 10 KHz was employed and 4K data points were collected. Exponential multiplication corresponding to 100Hz line broadening was applied during signal enhancement. 85% H_3PO_4 in 10% D_2O was used as an external standard.

3.5. Freeze-Fracture Electron Microscopy

Samples for freeze-fracture electron microscopy contained 30% v/v glycerol as a cryoprotectant. The lipids precipitated by acid or calcium ion were first spun down on an Eppendorf centrifuge, and pellets were mixed with equal volume of 60% glycerol in the given buffer. Final samples of less than 300µl were incubated in a water bath at the designated temperature for at least 10 mins, and were then quickly placed on gold cups which were on a hot plate at a temperature 5°C higher than that of the waterbath. The cups were immediately frozen in Freon 22 and stored in liquid nitrogen. Samples were fractured in a Balzer BA 360M Freeze Etch Unit at -115°C under a vacuum below 10^{-6} Torr, and replicated with carbon-platinum and then with carbon. Replicas were floated on water , washed for 60 mins in commercial Chlorax, twice in water, and mounted on bare 75X300 copper grids. The residual lipids were removed by placing over boiling chloroform and allowing vapors to condense on

the grids (Vail and Stollery, 1978). Specimens were examined in a Siemens Elmiskop 1A electron microscope.

4. Results

4.1. Aqueous Dispersions of TPE

The dispersion of transesterified egg phosphatidylethanolamine (TPE) in Tris-NaCl buffer(pH 7.4) precipitated rapidly to form large aggregates. Similar observations have been reported on egg phosphatidylethanolamine (Papahadjopoulos and Miller, 1967; Litman, 1973), dimyristoylphosphatidylethnaolamine and dipalmitoylphosphatidylethanolamine (Kolber and Haynes, 1979). In the DSC scan(Fig. III-1) TPE displayed a order-fluid transition at 15°C and a second transition at 56°C which has been correlated with bilayer-hexagonal transition (Boggs et al, 1981). The transition temperature of TPE is highly dependent on the source, a difference of 2°C in the main transition and 6°C in hexagonal transition has been observed with different batches of TPE. Since all TPE were shown to be free of oxidized products on thin layer chromatography, the difference in the transition are most likely due to slight variation in the acyl chain composition of natural phospholipids. This might explain the discrepancy in the transition temperatures reported from different laboratories (Boggs et al., 1981; Mantsch et al., 1981). The ^{31}P-NMR spectrum characteristic

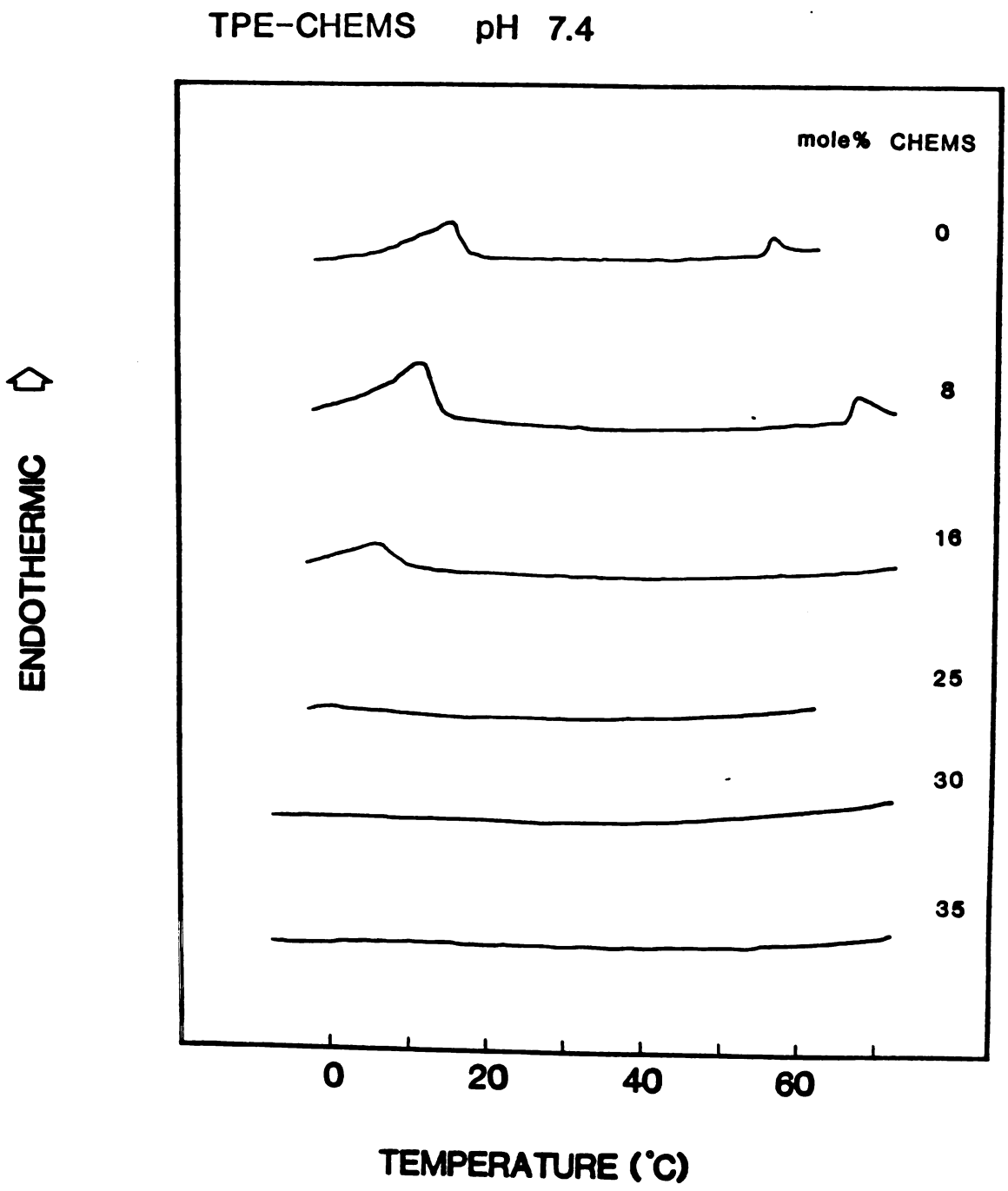


Figure III-1. Differential scanning calorimetry thermograms of the TPE-CHEMS mixtures at pH 7.4 with 0, 8, 16, 25, 30, 35 mole% of CHEMS.

of a lamellar phase was converted into a spectrum of a hexagonal phase in the temperature range corresponding to the second transition peak on the thermoscan (Fig. III-2). However, attempts to capture the hexagonal phase of TPE on freeze-fracture EM failed even when the temperature was raised to 80°C. A similar observation has been reported before (Hui et al., 1981a) and could be due to the difficulties in preserving the hexagonal structure at high temperature (Verkleij and de Gier, 1981). The freeze-fracture EM of TPE at 65°C showed fused lamellar structures with some in a concentric form(Fig. III-3, a).

4.2. Stabilization of TPE in Vesicular Form by CHEMS at Neutral pH

The addition of CHEMS to TPE altered the main transition of TPE in a similar fashion to that observed previously for DMPE-CHEMS mixtures (Chapter II). The incorporation of 8 mole% CHEMS decreased the main transition by 4°C; increasing the molar ratio of CHEMS shifted the order-fluid transition to lower temperature (Fig. III-1). The main transition was barely detectable with 25 mol% CHEMS added, and was completely suppressed as the content of CHEMS increased to 30 mole% (Fig. III-1). The opposite trend was seen on the lamellar-hexagonal transition of TPE as the CHEMS mole ratio was increased. At 8 mol% CHEMS, the peak temperature of L-H transition was moved to 67°C, 11°C higher than the hexagonal formation temperature of TPE (Fig. III-1). At greater

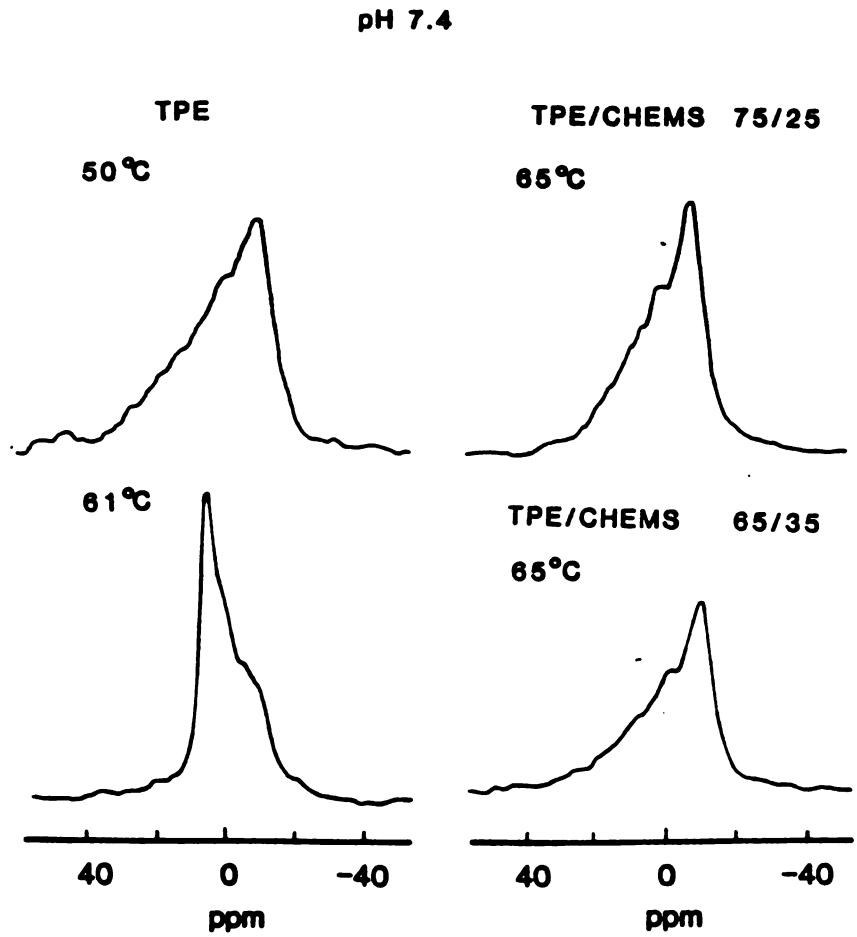


Figure III-2. Proton-decoupled ^{31}P-NMR spectra at 40.5 MHz of the aqueous dispersions of TPE and TPE-CHEMS at pH 7.4.

ratios of CHEMS, the L - H transition was not detectable even up to 72°C (Fig. III-1), which is also confirmed by the NMR spectra (Fig. III-2, TPE/CHEMS of 75/25 and 58/42 at 65°C). Multilamellar vesicles of TPE can be easily prepared with 20 mole% or more of CHEMS. Freeze-fracture EM showed that the vesicular structure was retained at temperatures above the hexagonal transition temperature of TPE (Fig. III-3, TPE/CHEMS(75/25) at 65°C) with the appearance of a few lipidic particles. Reverse phase evaporation vesicles (Ellens et al., 1984a) and small unilamellar vesicles were also readily prepared at these molar ratios (electron micrography not shown). These results are summarized in Table III-1.

4.3. Effect of pH on CHEMS/PE Vesicles

The CHEMS-TPE membranes were acidified to study the effect of protonation of the carboxylate group of CHEMS. TPE by itself is insensitive to the pH change in this region (Fig. III-4; Trauble and Eibl, 1974). Both the order-fluid and lamellar-hexagonal transitions of TPE were little affected when the pH was shifted to 4.5 (Fig. III-4). Figure III-5 shows the ^{31}P-NMR spectra of TPE at pH 4.5, confirming that the L - H transition occurred in the same temperature region. The change of pH from 7.4 to 4.5 also had a minimum effect on the main transitions of the TPE/CHEMS mixtures (Fig. III-4); the shift of the peak transition temperatures were less than 0.5°C. Since proton-induced phase

Figure III-3. Freeze-fracture electron microscopy of the aqueous dispersions of TPE and TPE/CHEMS at pH 7.4. (a) TPE at 65°C. (b) TPE/CHEMS(75/25) at 65°C. Bar is 0.2 µm. Arrows in (b) indicate lipidic particles.


a
b

TABLE III-1

Summary of Results

Mole% CHEMS in TPE	pH 7.4 DSC T_m (°C)	pH 7.4 DSC T_h	pH 7.4 ^{31}P-NMR L-H (°C)	pH 7.4 EM[a]	pH 4.5 DSC T_m	pH 4.5 DSC T_h	pH 4.5 ^{31}P-NMR L-H (°C)	pH 4.5 EM	3mM Ca^{++} pH 7.4 DSC T_m	3mM Ca^{++} pH 7.4 DSC T_h	3mM Ca^{++} pH 7.4 ^{31}P-NMR L-H (°C)	3mM Ca^{++} pH 7.4 EM
0	15	56	50-60	L*, 65°C not V*	15	56	50-60	--	15[b]	56[b]	--	--
8	11	67	--	--	11	43	--	--	--	--	--	--
16	7	>72	--	--	7	34	--	--	--	--	--	--
25	3	>72	>65	V, 65°C	3	26	18-30	H*, 37°C H, 22°C AV*, 4°C	--	--	--	--
30	ND[c]	>72	--	V, 65°C[b]	ND	17-28	--	--	ND[b]	>72[b]	66-78	AV, 25°C
35	ND	>72	>65	--	ND	ND	5-18	--				
42	ND	>72	>65	V, 65°C[b]	ND[b]	ND[b]	--	H, 22°C				
8 + 30% glycerol	--	--	--		12	33	--					

a EM samples contain 30% v/v glycerol.
b Data not shown.
c ND, not detectable.
* L, lamellar structure; V, vesicles; AV, aggregated vesicles; H, hexagonal phase.

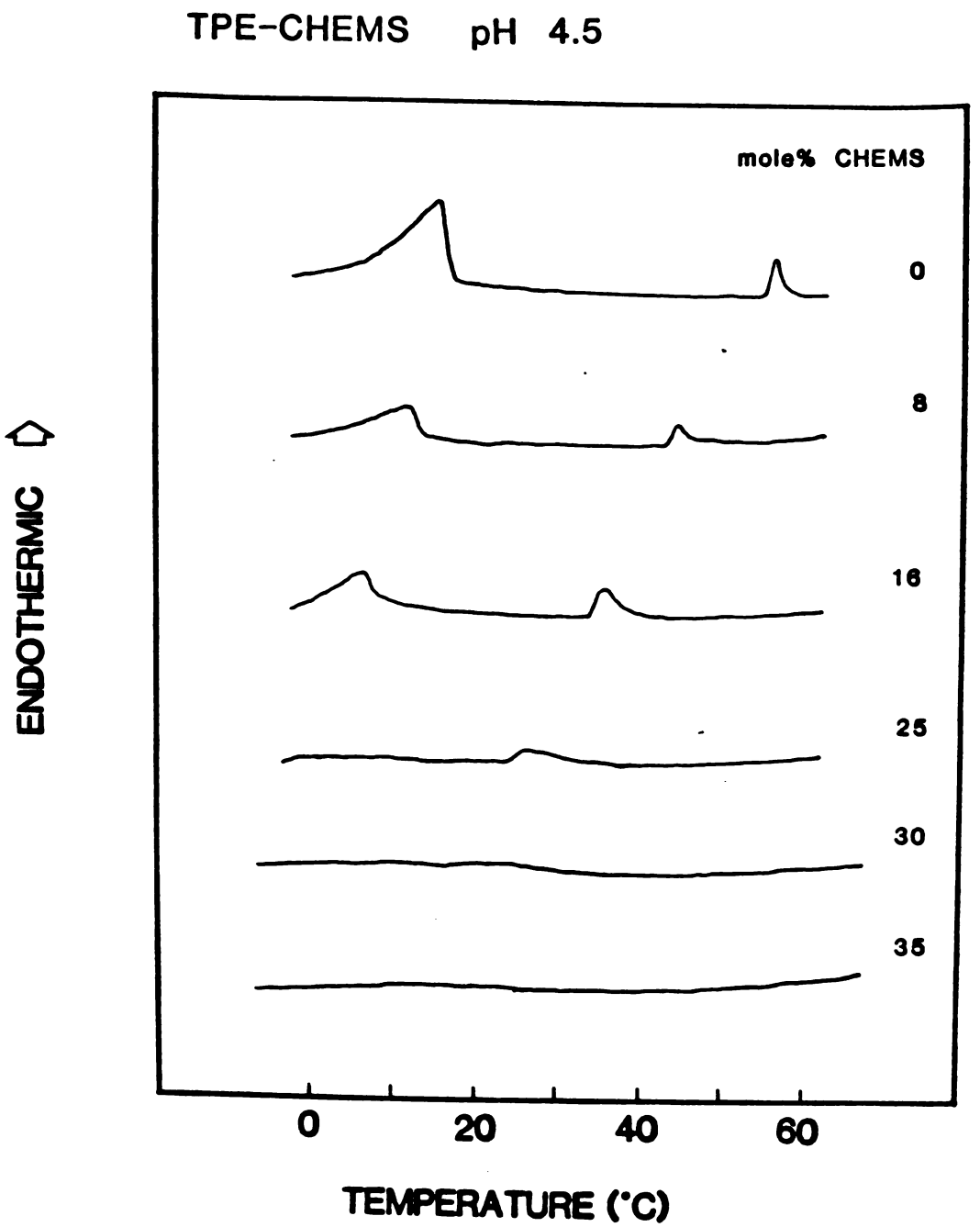


Figure III-4. Differential scanning calorimetry thermoscan of the TPE-CHEMS mixtures at pH 4.5 with 0, 8, 16, 25, 30, 35 mole% of CHEMS.

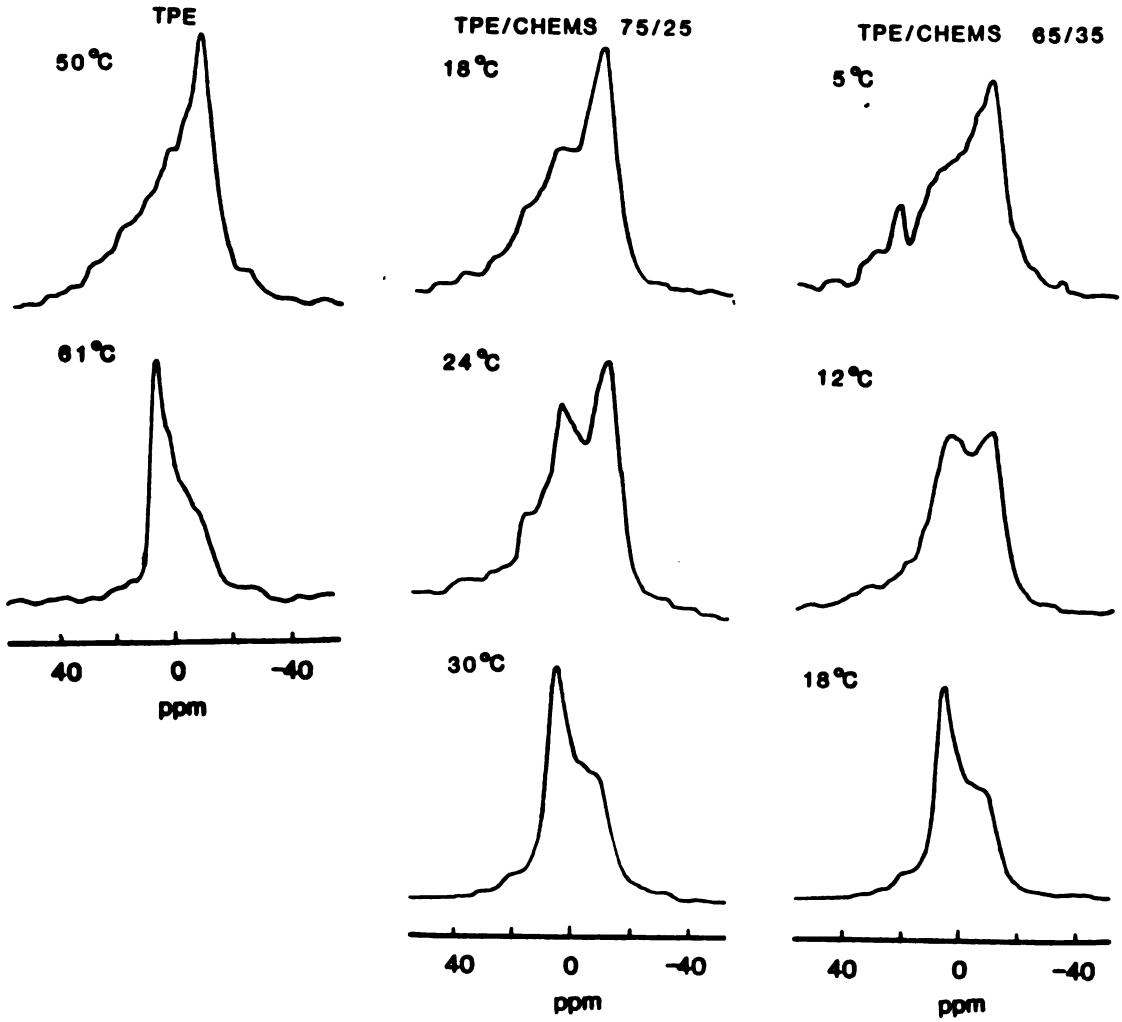


Figure III-5. Proton-decoupled ^{31}P-NMR spectra at 40.5 MHz of TPE and TPE-CHEMS at pH 4.5.

separation of PC/PS membranes occurs slowly with an equilibrium time of 2 hour at pH 2.5 (Tokutomi et al., 1980), to determine if the phase separation of TPE/CHEMS (70/30) is also a slow process, we allowed the system to equilibrate for 24hr at low pH. Under this condition the scan was identical to that before equilibrium. On the other hand, the L - H transitions of TPE/CHEMS mixtures were highly acid-sensitive. For TPE/CHEMS of 92/8 composition at pH 4.5, the peak hexagonal transition temperature was shifted to 43°C (Fig. III-4), a 24°C downshift from the corresponding transition peak at neutral pH, and 13°C lower than that of pure TPE. The shift in the L - H transition was dependent on the acid concentration; the transition temperature at pH 4.5 is 5°C and 1°C, respectively, lower than that at pH 5.5 and 5.0 (Fig. III-6), implying that the promotion of the H_{II} phase is related to the protonation of CHEMS. In addition, the more CHEMS in the TPE mixtures, the lower the temperature at which the acid induced hexagonal transition takes place (Fig. III-4). For TPE containing 25 mole% CHEMS the peak hexagonal transition temperature is 26°C at pH 4.5, a temperature that is 30°C lower than the L - H transition temperature of TPE. ^{31}P-NMR spectra also indicated a hexagonal transition in this temperature region (Fig. III-5, TPE/CHEMS at 75/25). The presence of the hexagonal phase at 37°C was confirmed by freeze-fracture EM (Fig. III-7, a), showing stacked lipid cylinders running parallel to each other. The L - H transition temperatures of the mixtures in

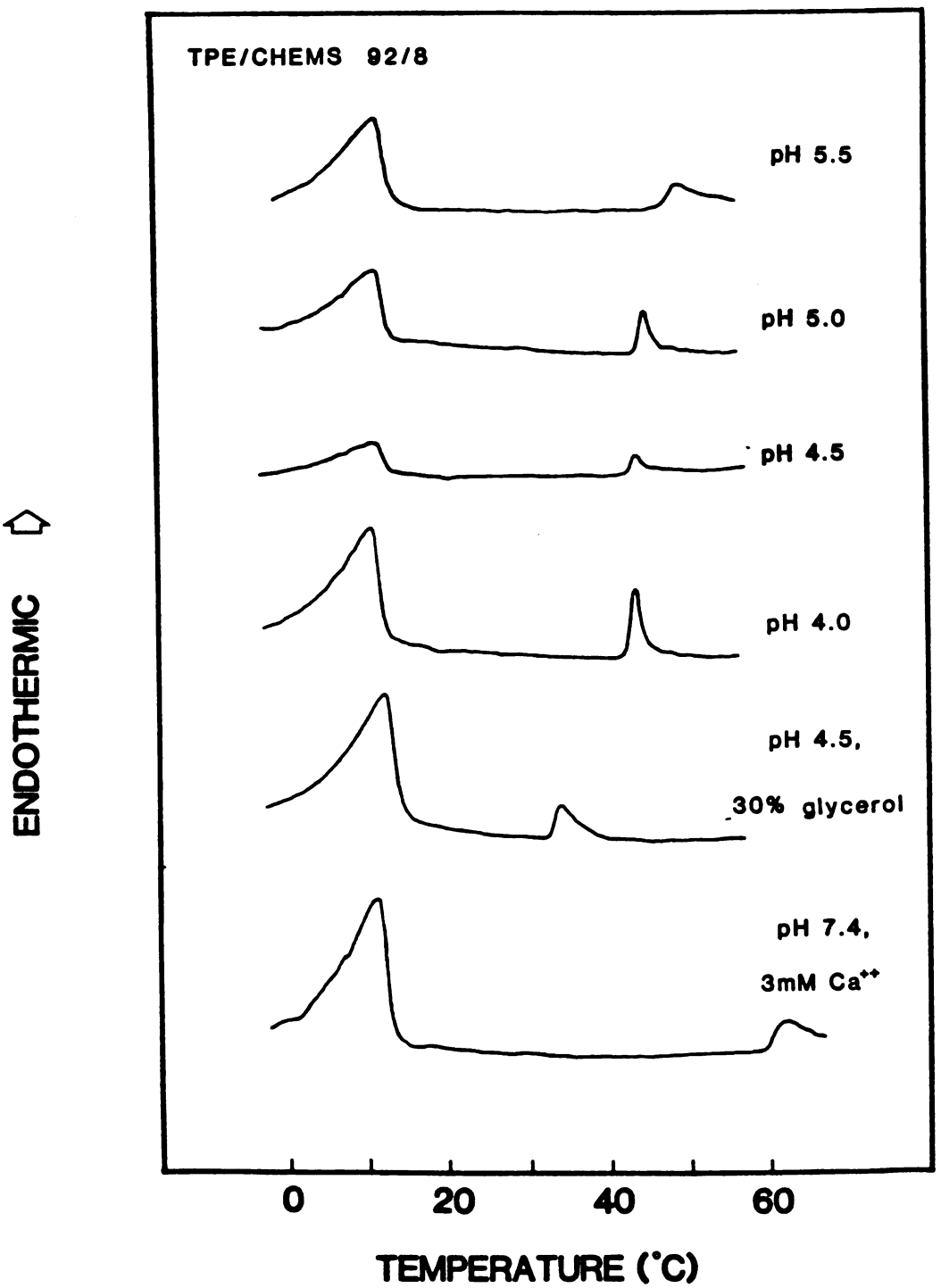


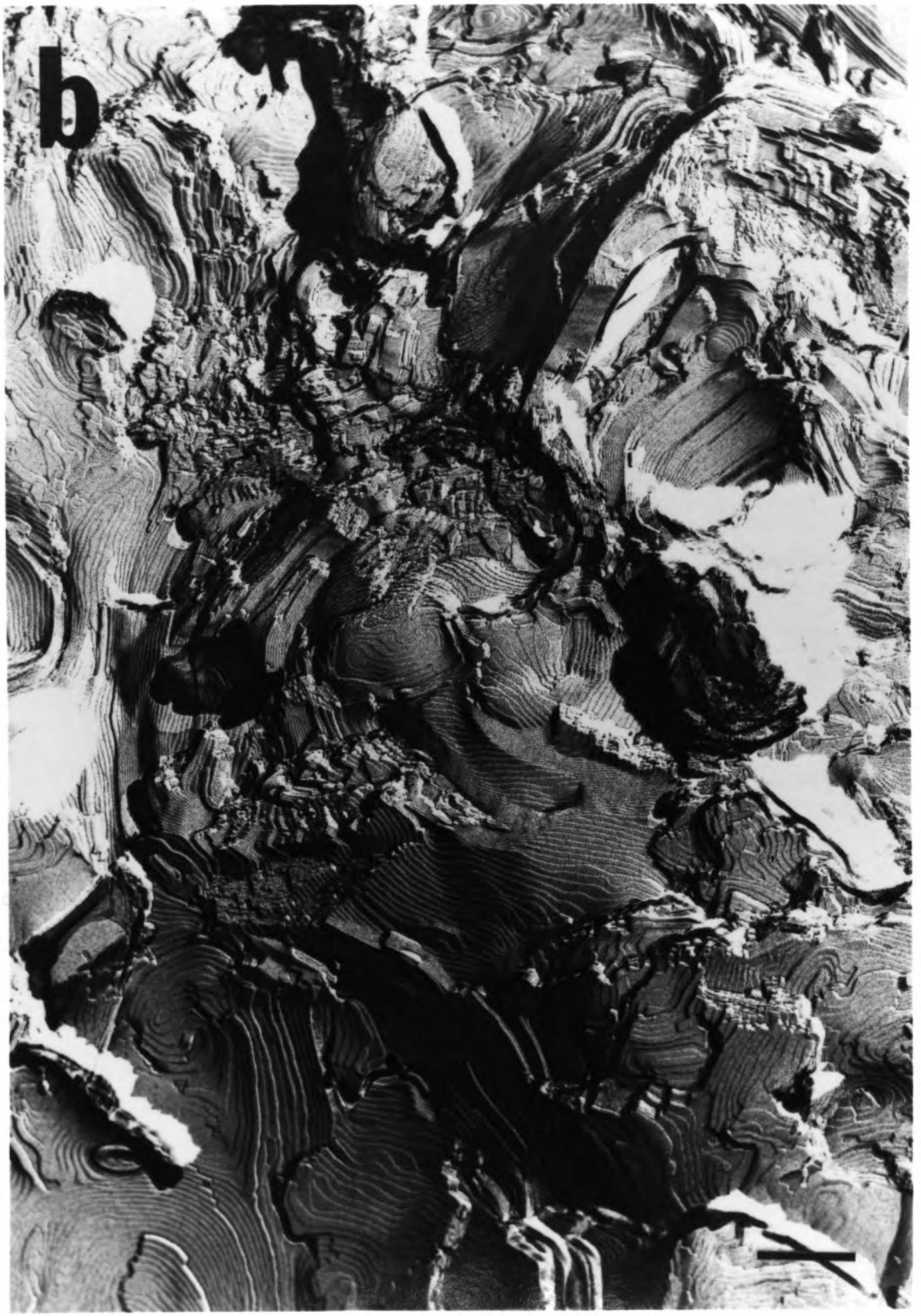
Figure III-6. Thermoscans of TPE-CHEMS(92/8) at pH 5.5, pH 5.0, pH 4.5, pH 4.0; pH 4.5 with 30% glycerol; and pH 7.4 with 3mM Ca^{++}.

30% glycerol (for freeze fracture), however, were always 8-11°C lower than the same mixture in the absence of glycerol. At 22°C the acidified TPE/CHEMS (75/25) remained in hexagonal and hexagonal-like structures (Fig. III-7, b). Electron microscopy of lamellar structures could be observed only when the TPE-CHEMS mixture was incubated at temperatures below 10°C. Fig. III-7c shows the structure of aggregated vesicles of TPE/CHEMS (75/25) at 4°C and pH 4.5. Such a H_{II} promoting effect by glycerol can be easily observed in the thermoscan of TPE with 8 mole% CHEMS (Fig. III-6, at 30% glycerol). Similar shifts in the endothermic H_{II} transition were found with TPE/CHEMS at 82/16 and 75/25 compositions containing 30% glycerol (scan not shown).

At 30 mole% CHEMS the endothermic L - H transition induced by acid was barely detectable between 17°C and 28°C (Fig. III-4). The acid-induced hexagonal transition was not detectable on DSC as the content of CHEMS in TPE mixtures reached 35 mole% (Fig. III-4). NMR spectra indicated that the lipid mixture still underwent a lamellar-hexagonal transition at temperatures between 5 and 18°C(Fig. III-5, TPE/CHEMS at 65/35). The hexagonal structure was also demonstrated by freeze-fracture EM for TPE containing higher amounts of CHEMS(Fig. III-7, d). The acid-induced hexagonal phase formation is summarized in Table III-1.

The membrane stabilization effect of CHEMS was also studied with DOPE. DOPE has a hexagonal transition tempera-

Figure III-7. Freeze-fracture electron microscopy of the lipids at pH 4.5. (a) TPE/CHEMS(75/25) at 37°C. (b) TPE/CHEMS(75/25) at 22°C. (c) TPE/CHEMS(75/25) at 10°C. (d) TPE/CHEMS(58/42) at 20°C. (e) EPC/CHEMS(70/30) at 65°C. Bar is 0.2 μm.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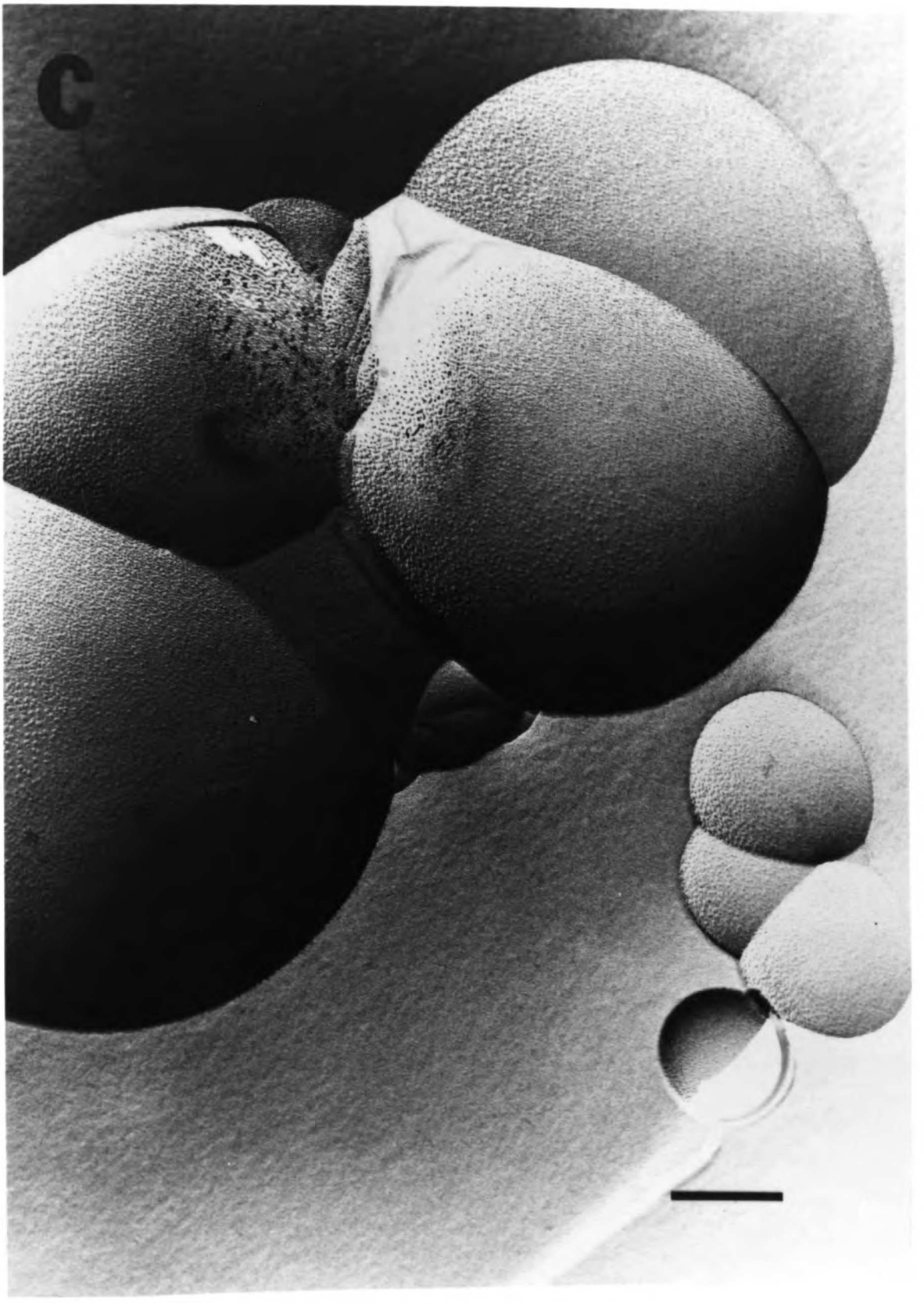
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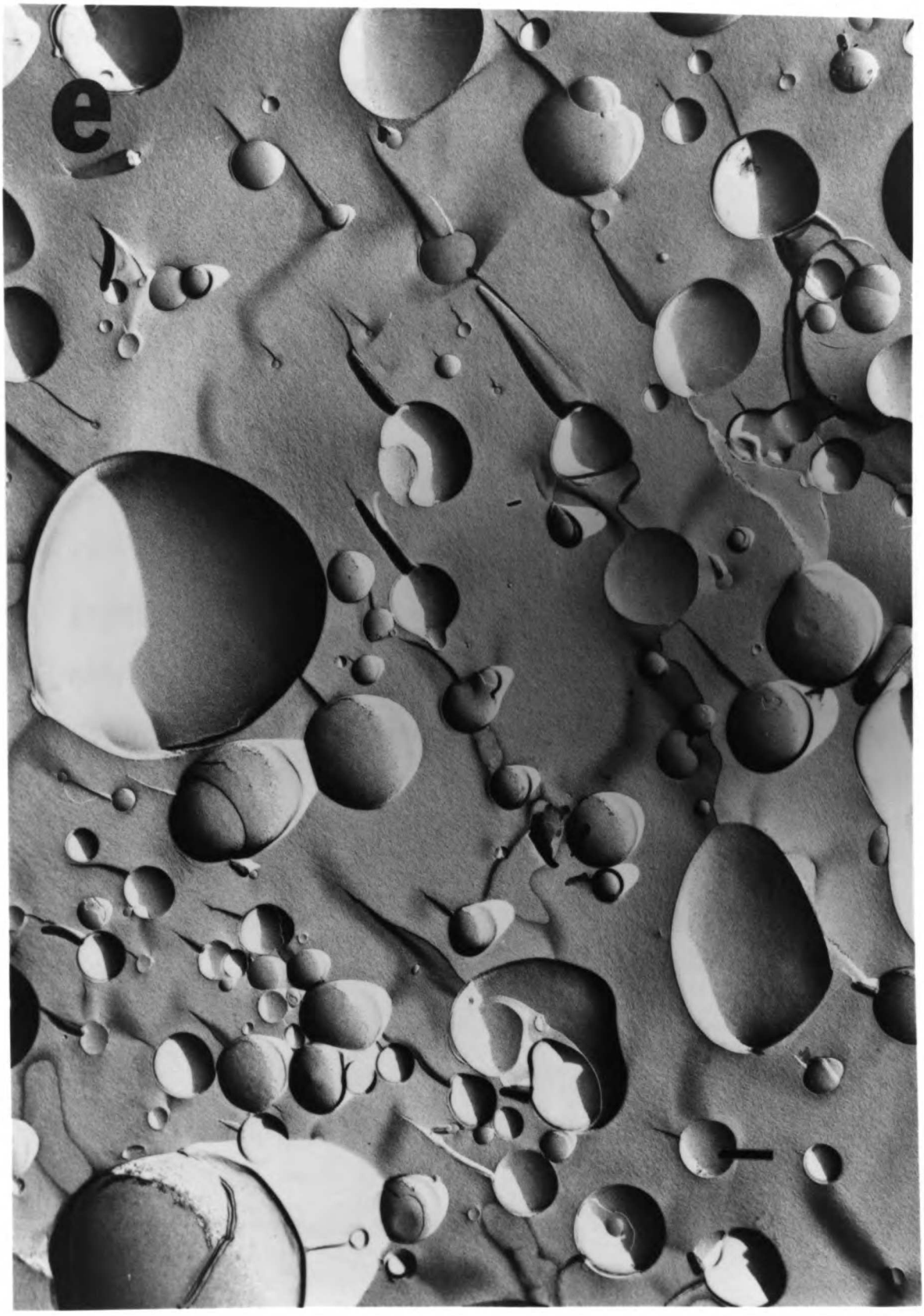

e

ture in the range of 10-18°C (Cullis and de Kruijff, 1976). At 25°C the addition of CHEMS in excess of 20 mol% stabilized DOPE in the vesicle form. Acidification of the vesicles resulted in the reappearance of the hexagonal phase as detected by freeze-fracture EM and ^{31}P-NMR (data not shown).

In contrast to PE membranes, PC membranes containing CHEMS were only slightly affected by acidification. Upon exposure to pH 4.5 buffer, liposomes composed of EPC/CHEMS(7/3) slowly aggregated. The aggregates could be redispersed and freeze-fracture EM demonstrated the dispersion remained in the form of vesicles (Fig. III-7, e).

4.4. Effect of Calcium on CHEMS/PE Mixtures

Addition of calcium ions to TPE/CHEMS(7/3) liposomes also aggregated the vesicles and released the contents of the vesicles in a way similar to the low pH effect (Ellens et al., 1984b). Examination of the aggregated lipid with ^{31}P-NMR indicated that the mixture was in the lamellar phase at 25°C (Fig. III-8). The ^{31}P-NMR spectra characteristic of a lamellar phase predominated even on heating to 65°C (Fig. III-8), the temperature at which TPE should be in a hexagonal phase. The hexagonal phase did not appear until 78°C (Fig. III-8). The freeze-fracture EM showed aggregated vesicles, some of much larger diameter (Fig. III-9, b) than the original preparation (Fig. III-9, a). The Ca^{++}-aggregated structures are in contrast to the H_{II} structures of the TPE-CHEMS mixtures of similar compositions at the

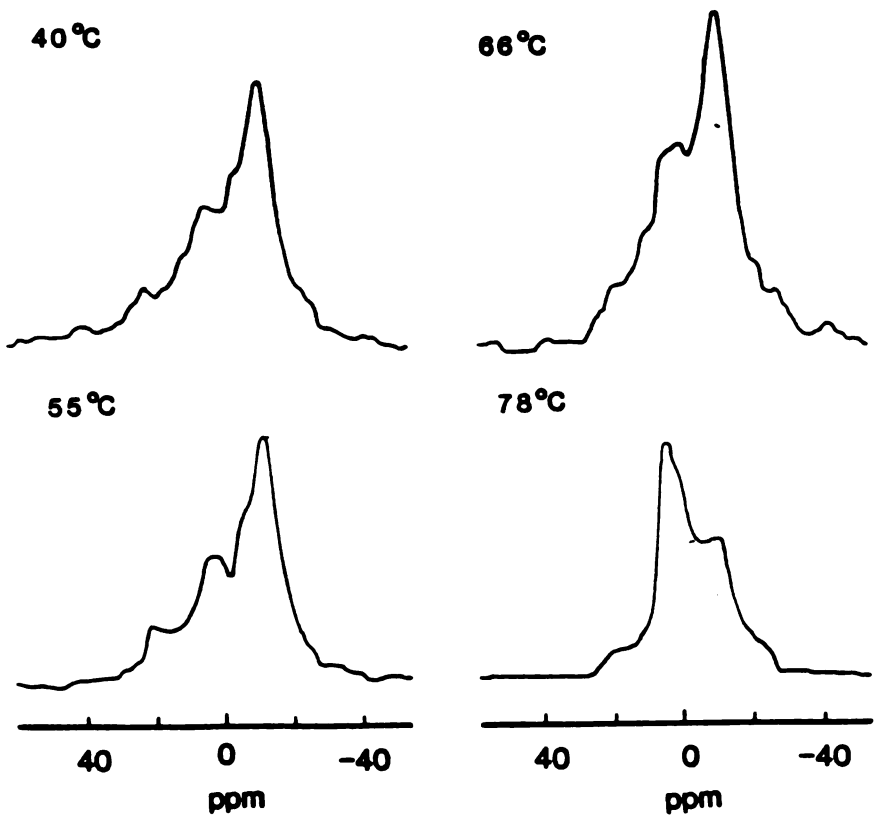


Figure III-8. Proton-decoupled ^{31}P-NMR spectra at 40.5 MHz of TPE/CHEMS(70/30) aggregated by dialysis against 3 mM Ca^{++}.

same temperature (Fig III-7, c,e). The thermoscan of this mixture (scan not shown) is identical to that in the absence of Ca^{++} (Fig. III-1, TPE with 30% CHEMS) between $-8^{o}C$ and $72^{o}C$. The main transition of TPE remained suppressed in the presence of Ca^{++}, indicating that macroscopic phase separation did not ensue. The effect of Ca^{++} can also be seen in the DSC scan of TPE/CHEMS(92/8) (Fig. III-6). In the presence of 3mM Ca^{++}, the hexagonal transition temperature of the lipid mixture decreased from $67^{o}C$ to $62^{o}C$, which is still $6^{o}C$ above the H_{II} formation temperature of TPE. The effect of Ca^{++} on TPE/CHEMS is also summarized in Table III-1.

Ca^{++} caused similar changes to DOPE/CHEMS(7/3) vesicles: liposomes aggregated by 5mM Ca^{++} had a hexagonal transition temperature of $30\text{-}37^{o}C$, almost $20^{o}C$ higher than that of pure DOPE (NMR spectra not shown).

5. Discussion

5.1. Interaction of CHEMS with TPE at Neutral pH

Although the 3β-OH of CHEMS is esterified, CHEMS retains the affinity of cholesterol for phospholipids (Simmonds et al., 1984; Chapter II). The interaction of CHEMS with DMPE, leading to the broadening and eventual suppression of the main transition of DMPE, is indistinguishable from that of cholesterol (Chapter II). Similar results were

Figure III-9. Freeze-fracture electron microscopy of TPE/CHEMS(70/30) (prepared by reverse-phase evaporation method, 0.2 μ extruded). (a) before, (b) after the dialysis against 3 mM Ca^{++}, 25°C. Bar is 0.2 μm.


a
b

demonstrated with TPE-CHEMS in this Chapter. Owing to the heterogeneous acyl chain composition, the order-fluid transition of TPE proceeds in a less cooperative way than DMPE as indicated by the width of the main transition peak. A lower content of CHEMS (30 mole%) was enough to broaden the main transition of TPE so that it was no longer detectable by DSC.

PE vesicles are difficult to prepare at neutral pH. For PE with highly unsaturated acyl chains, the integrity of the bilayer surface cannot be maintained due to the H_{II} phase formation at low temperature (Cullis and de Kruijff, 1979; Mantsch et al., 1981). For more saturated PEs which do not undergo the hexagonal transition until high temperature (Harlos and Eibl, 1981; Seddon et al., 1983), aggregates of lamellar structure are formed at pH 7.0 (Kolber and Haynes, 1979). These properties have been attributed to the conformation of the phosphorylethanolamine head groups which allows a strong intermolecular interaction between the ammonium and phosphate groups of adjacent PEs (Hitchcock et al., 1974; Hauser et al., 1981). In the present study we have demonstrated that the incorporation of CHEMS stablizes PE in bilayer membranes. The stablization effect of CHEMS is manifest in both the inhibition of the lamellar to hexagonal phase transition and in the formation of bilayer vesicles of PE. The incorporation of 8 mole% CHEMS raises the L - H transition temperature of TPE by 11°C (Fig. III-1),

whereas additional CHEMS moves the hexagonal transition beyond 72°C (Fig III-1, TPE with 16-35 mole% CHEMS). When the mole ratio of CHEMS exceeds 20%, TPE forms stable vesicles. Similar results were observed for DMPE (Chapter II) and DOPE. The mole ratio of CHEMS required to stablize PE in a bilayer is in the same range as PC on PE membranes (Hui et al., 1981a; Tilcock et al, 1982; Dekker et al, 1983; Boni and Hui, 1983). The membrane-stabilization effect of CHEMS is probably due to the disruption of the intermolecular interaction between adjacent PEs, in a fashion similar to other phospholipids (Kolber and Haynes, 1979; Hui et al., 1981a, Tilcock et al., 1982) or that observed when PE is deprotonated by raising the pH (Stollery and Vail, 1977).

5.2. Effect of pH on CHEMS/PE Mixtures

The protonation of CHEMS immediately leads to the aggregation of TPE/CHEMS and DOPE/CHEMS liposomes with the leaking of the encapsulated contents(Ellens et al., 1984b). Of particular interest is that the L - H transition is dramatically lowered, while the main transition of TPE/CHEMS is not affected by the protonation of CHEMS. AT 30 mole% CHEMS, the order-fluid transition of TPE was suppressed at pH 4.5 (Fig. III-4). Even after a 24 hr incubation at pH 4.5 at temperature above the L - H transition the main transition of TPE was not detectable. This implies that protonated CHEMS remains effectively complexed with phosphatidylethanolamine. CHEMS is therefore different from acidic

phospholipids in that macroscopic lateral phase separation does not occur following the protonation of the charged group.

Lateral phase separation occurs when the clustering of each type of lipid is energetically favored over the mixture of the lipids (Trauble, 1976). The association of protonated CHEMS with TPE is therefore more stable than the segregation of CHEMS from TPE. In Chapter II we have shown that 3β-OH of cholesterol is not required for the suppression of phospholipid order-fluid transition. Here we have also demonstrated that phase transition suppression persists even after titration of the carboxylate group. It is therefore unlikely that the interaction of CHEMS and phospholipid involves hydrogen bonding between the carboxylate group of CHEMS and a polar component of phospholipid. Since the orientation of the sterol in the phospholipid membranes determines the extent of sterol-phospholipid interaction (Cadenhead and Muller-Landau, 1979; Chapter II), the results here suggest that the protonated CHEMS remains in a configuration that allows the maximum van der Waals interaction between sterol and phospholipid.

The hexagonal phase is adopted by a lipid when the area of the acyl chain region exceeds the head group area (Cullis and de Kruijff, 1979; Israelachvili et al., 1980; Mantsch et al., 1981). The association of column-shaped lipid with cholesterol has been suggested to produce a conically-shaped

molecule that prefers the hexagonal phase (Cullis and de Kruijff, 1979; Wieslander et al., 1980). We believe the tight association of CHEMS with TPE promotes the "conical-shape" TPE complex as CHEMS becomes uncharged. This is illustrated in Fig. III-10(a): the protonation of CHEMS favors the negatively-curved surface of the hexagonal phase. It may be noted that the H_{II} promoting effect of protonated CHEMS is unusually high compared to that of other sterols (Gallay and de Kruijff, 1982; Gallay et al, 1984). While the addition of 50 mole% cholesterol to PE decreases the hexagonal transition temperature by only 10-20°C (Cullis and de Kruijff, 1978b; Dekker et al, 1983), the incorporation of 25 mole% CHEMS reduced the L - H transition temperature of TPE by 30°C at pH 4.5. Since the extent of interaction with phospholipid is almost the same for CHEMS and cholesterol (Chapter II, Simmonds et al, 1984), the greater H_{II}-promoting effect should be due to the presence of the succinyl ester. The exact molecular mechanism involved requires further elucidation.

The endothermic peak of the L - H transition of TPE is broadened by the addition of CHEMS. An interesting finding is that the acid-induced H_{II} endothermal peak is suppressed as the molar ratio of CHEMS in TPE exceeds 35% (Fig. III-4) even though the ^{31}P-NMR spectra indicate that a L - H transition still occurs (Fig. III-5, TPE/CHEMS at 65/35). This transition is observed in a temperature interval of 10-15°C,

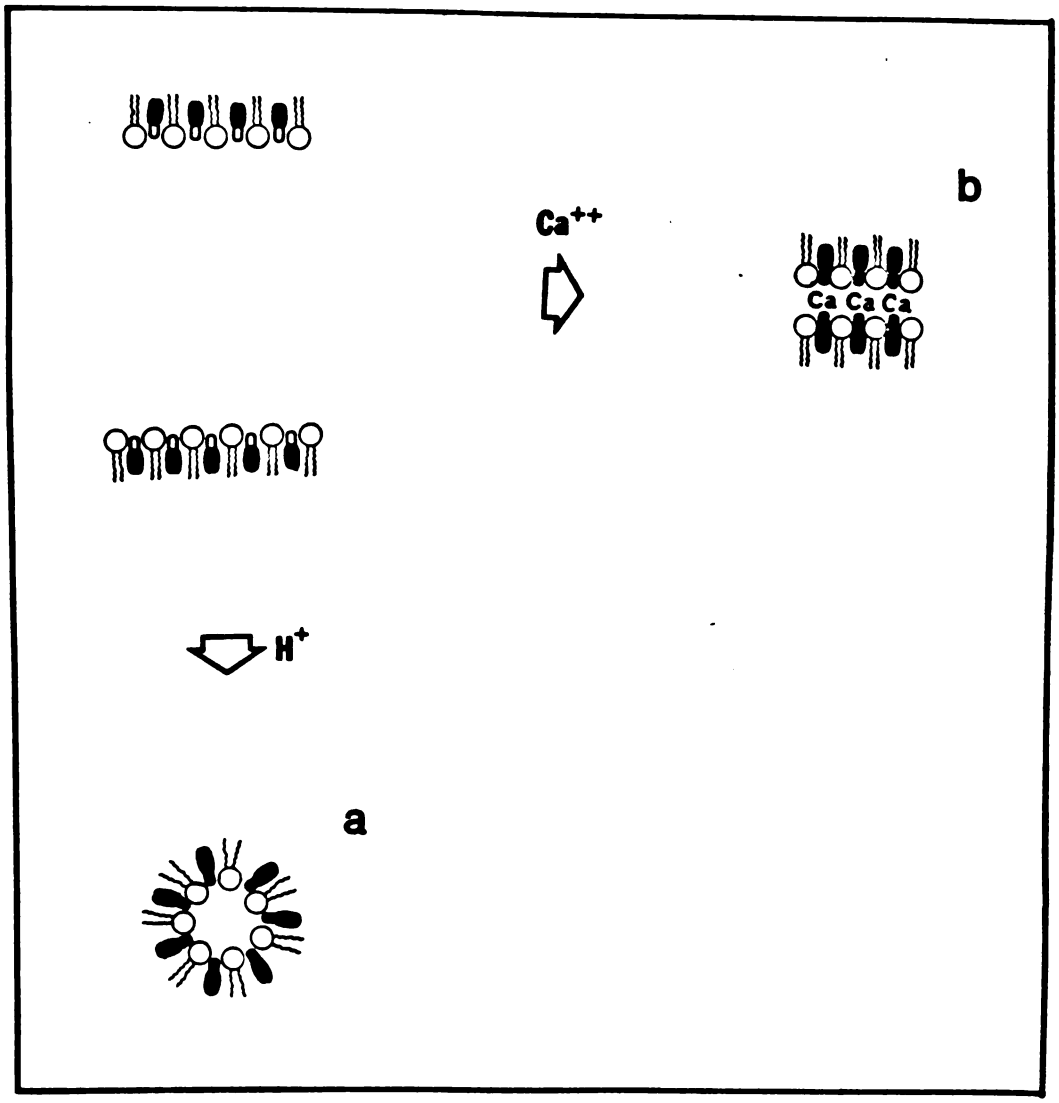


Figure III-10. Pictorial representation of the interactions of TPE-CHEMS with protons and calcium ions. , TPE; , CHEMS. (a) Protons neutralized CHEMS remain complexing with TPE, and promote the formation of hexagonal phase. (b) Calcium form bridge between CHEMS of opposite bilayers, resulting in a rigid lamellar structure that prevent TPE to go into hexagonal phase.

implying that the hexagonal transition is still cooperative. The suppression of the endothermic H_{II} transition, therefore, is different from the suppression of the main transition of phospholipids by sterols. A possibility is that after sufficient amounts of CHEMS intercalate between PE molecules, the energy barrier between the lamellar and hexagonal phases of TPE-CHEMS becomes too small to be detectable by calorimetry as CHEMS is titrated. This is also the case for PE with homogeneous unsaturated acyl chains, such as DOPE, in which the L - H transition is not detectable by DSC (van Dijck et al., 1976; Cullis and de Kruijff, 1979).

5.3. Effect of Calcium on CHEMS/PE Membranes

Both Ca^{++} ions and protons induce the order-fluid transition of acidic phospholipids through charge neutralization (Trauble and Eibl, 1974; Jacobson and Papahadjopoulos, 1975; Trauble, 1976). Calcium ions also induce phase separation of bilayers containing PS and PA (Jacobson and Papahadjopoulos, 1975; van Dijck et al., 1978). While PE is only slightly affected by the presence of Ca^{++} at neutral pH (Harlos and Eibl, 1980b), its mixture with CHEMS is very sensitive to Ca^{++}. The TPE/CHEMS vesicles are readily aggregated upon the addition of calcium. The examination of lipid aggregates by EM showed fused structures that have a ^{31}P-NMR characteristic of a lamellar phase. NMR spectra indicated the L - H transition of Ca^{++}-(TPE/CHEMS) occurs at a temperature higher than the H_{II} formation temperature of

TPE (Fig. III-8). In contrast to the H_{II} promoting effect of protons, Ca^{++} seems to stabilize TPE in the lamellar phase when CHEMS is present. It is well known that calcium ions and protons have different effects on the phase transition of acidic phospholipids. The addition of Ca^{++} shifts the order-fluid transition to a much higher temperature than protonation (Jacobson and Papahadjopoulos, 1975; van Dijck, 1978; Liao and Prestegard, 1981). Based on these observations, Jacobson and Papahadjopoulos (1975) have suggested that the addition of Ca^{++} to anionic phospholipids involves more than charge neutralization. X-ray diffraction indicates that calcium addition results in a close opposition of PS bilayers (Newton et al., 1978). Similar complexes of Ca^{++}-PG (Harlos and Eibl, 1980a) and Ca^{++}-PA (Liao and Prestegard, 1981) have also been characterized. Portis et al. (1979) proposed a trans-complex between Ca^{++} and the PS head group in which Ca^{++} ions form a bridge between apposed bilayers. A study on the metastable phase transition of Ca^{++}-PG led to a similar model (Boggs and Rangaraj, 1983). McLaughlin (1982) has demonstrated that the carboxylate group is the major site in PS where divalent cations form a tight complex. We propose a similar type of crystallization of CHEMS by Ca^{++} (Fig. III-10, b). In this diagram Ca^{++} neutralizes the negative charge of CHEMS and forms a trans-bridge between two adjacent bilayers. The rigid lattice of Ca^{++}-CHEMS holds TPE in the lamellar phase even at temperatures above the hexagonal transition temperature of TPE.

There is, however, one major difference between calcium-CHEMS and calcium-acidic phospholipids complex: the complexation of CHEMS with Ca^{++} does not induce macroscopic phase separation in TPE/CHEMS membrane systems as indicated by DSC scans. The observations that the charge neutralization by both H^{+} and Ca^{++} does not lead to extensive lateral phase separation reflects the more specific interaction between CHEMS and neutral phospholipids, which is lacking between acidic phospholipids and neutral phospholipids. Because of this strong association, titration of the succinyl group with protons results in the enhancement of H_{II} phase in TPE. On the other hand, when CHEMS is chelated by calcium to form a stable lamellar structure, the TPE is also immobilized in the lamellar phase.

5.4. Possible Biological Significance

The presence of H_{II} phase lipid has been implicated in several biological functions such as membrane fusion, transbilayer transport (Cullis and de Kruijff, 1979) and membrane enzyme activity (Madden et al., 1983). The presence of the acidic cholesterol ester inhibits the formation of the hexagonal phase and stabilizes PE in bilayer membranes at neutral pH. This might be the reason for the observed membrane stabilization effect of cholesteryl sulfate on erythrocytes and spermatoza (Beau et al., 1974; Langlais, 1981) and for the pathological state in the skin of patients with recessive X-linked ichthyosis (Williams and Elias, 1981). We

have demonstrated here that the properties of a model membrane can be regulated by charge neutralization. Similar effects could occur in biological membranes at high levels of calcium or by enzymatic removal of the sulfate or other groups. Although a direct relationship between biofunction and cholesteryl sulfate remain to be established, the present study provides a structural basis for how such effects could originate.

6. Conclusion

In the present study we have used cholesterylhemisuccinate to demonstrate the unusual properties of anionic cholesterol esters. Charged cholesterylhemisuccinate is different from cholesterol in its capacity to stabilize PE in bilayer membranes and to prevent the formation of the hexagonal phase. The acidic cholesterol ester is also different from acidic phospholipids due to its specific interaction with phospholipids and its suppression of the order-fluid transition of the associated phospholipids. Upon neutralization by protons or Ca^{++}, cholesterylhemisuccinate retains a substantial affinity for the phospholipid and macroscopic phase separation does not ensue. The association of protonated cholesterol ester with phospholipid promotes the formation of a hexagonal phase, whereas the interaction of calcium-chelated CHEMS with PE may stabilize

PE in a lamellar phase. These findings point out the possible biological relevance of acidic cholesteryl esters in biomembranes.

CHAPTER IV

ACID-SENSITIVE PHASE TRANSITIONS IN DIPALMITOYLPHOSPHATIDYLCHOLINE - TOCOPHEROL ACID SUCCINATE MIXTURES

1. Summary

The incorporation of tocopherol acid succinate (TS) into dipalmitoylphosphatidylcholine (DPPC) membranes destabilized the phospholipid vesicle at pH 4.5. At 65°C DPPC with 40 mol% TS had a ^{31}P-NMR spectrum indicating an isotropic phase. Increasing the molar ratio of TS to 60% resulted in a ^{31}P-NMR spectrum characteristic of a hexagonal phase, while a mixture of bilayer and planar lamellar structures were clearly demonstrated by freeze-fracture electron microscopy and X-ray diffraction. Hexagonal-like structures could however be identified in the electron micrographs of samples containing 30% glycerol. In comparison, cholesterylhemisuccinate at 60 mol% had no effect on the lamellar structure of DPPC at 71°C and pH 4.5. All mixtures containing CHEMS or TS showed a lamellar phase at pH 7.4.

2. Introduction

Phosphatidylcholine is well known for its ability to form a stable bilayer structure over a wide range of temperatures, hydration states and ionic concentrations. This stability probably accounts for its predominance in most biological membranes. This is in contrast to the ability of certain phospholipids such as cardiolipin and phosphatidylethanolamines to form non-bilayer structures. The bilayer-preference of PC is attributed to the relatively large phosphorylcholine group which can accommodate the numerous arrangements of the hydrocarbon chains while maintaining an approximately constant head group to acyl chain area in the molecule (Hauser et al., 1981). Because of its unusual stability in lamellar structure, PC has been used in mixtures with lipids that form non-bilayer structures to study the structural changes that occur during transitions between bilayer and non-bilayer phases (de Kruijff et al., 1979; Hui et al., 1981a; Borovjagin et al., 1982; Boni and Hui, 1983).

Lipid polymorphism of phosphatidylcholine in the presence of excess water, however, has recently been reported. At pH 4.0 DPPC with 66 mol% palmitic acid is shown to have a direct transition from lamellar gel to hexagonal phase at 61^{o}C (Marsh and Seddon, 1982). Dekker et al. (1983) have also shown that the addition of equimolar cholesterol to highly unsaturated PC resulted in a ^{31}P-NMR spectrum of the isotropic or hexagonal type. In the present study toco-

pherol acid succinate (TS), a hydrophilic ester with a greater solubility than tocopherol in phospholipid membranes (Chapter II), was incorporated into DPPC at neutral pH. The protonation of TS by lowering the pH is shown to produce structures containing planar lamellar sheets. Although the ^{31}P-NMR spectrum generated is characteristic of the hexagonal phase, freeze-fracture electron microscopy and X-ray diffraction failed to detect any hexagonal structures.

3. Materials and Methods

Dipalmitoylphosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL). Tocopherol acid succinate and cholesterylhemisuccinate were obtained from Sigma. Tocopherol acid succinate was converted to its morpholine salt by mixing with equimolar morpholine in chloroform solution. All lipids were shown to be pure by thin layer chromatography and were stored under nitrogen at -50°C. Multilamellar vesicle dispersions were prepared in Tris-NaCl buffer (50 mM Tris, 110 mM NaCl, pH 7.4). Differential scanning calorimetric measurements were made with a Perkin-Elmer DSC-2 Calorimeter. ^{31}P-NMR spectra were recorded on a Varian XL-100 Spectrometer operating in Fourier transform mode at 40.5 MHz. Accumulation of free induction decays were obtained from 3,000-6,000 transients with a 45° pulse angle, 0.5 sec interpulse time and a delay of 200 μsec.

Samples containing 30% v/v glycerol were fractured in a Balzer BA 360M Freeze Etch Unit at -115°C. Specimens were examined in a Siemens Elmiskop 1A electron microscope. The detail procedures of each measurement were the same as described in Chapter III. Rapid-freezing procedures were used for samples without cryoprotectants (Epand et al., 1981). Samples were sandwiched between copper foils and plugged into liquid propane using a Guillotine type apparatus. Fracturing and replicating were performed in a Polaron E 7500 freeze fracture unit at -115°C. The replicas were viewed in a Siemens 101 microscope. The X-ray diffraction patterns were recorded on a Frank-type camera mounted on a Jarrell-Ash microfocusing X-ray unit; the temperature of the sample was kept constant during exposure (Hui et al., 1981a).

4. Results

TS can be readily incorporated into DPPC bilayers at neutral pH. Aqueous dispersions of DPPC-TS encapsulate aqueous markers such as ^{3}H-sucrose (Chapter II). Freeze-fracture electron microscopy confirmed that these dispersions are vesicular in appearance (not shown). ^{31}P-NMR spectra of DPPC with 40 mol% and 60 mol% TS at pH 7.4 are characteristic of a lamellar structure as shown in Fig. IV-1,c. Dialysis of DPPC-TS liposomes against pH 4.5 buffer

(acetate/acetic acid 50 mM, NaCl 110mM) resulted in the aggregation of the liposomes. For DPPC with 40 mol% TS, the aggregate at 35°C had a ^{31}P-NMR spectrum with a lower field shoulder and high field peak, indicating a lamellar structure (Fig. IV-1, a). X-ray diffraction of the same aggregates gave a series of broad reflections corresponding to a repeat spacing of 84 Å, characteristic of a lamellar phase of a negatively charged phospholipid. Freeze-fracture electron microscopy showed the uneven fracture faces of multilamellar vesicles (Fig. IV-2, a). At elevated temperatures a combination of a narrow symmetric peaks and a spectrum characteristic of a lamellar phase was obtained by ^{31}P-NMR (Fig. IV-1, a). A further increase of temperature was accompanied by a decrease in the lamellar-phase signal and an increase in an isotropic signal centered at 0 ppm. At 65°C an exclusive isotropic NMR signal was obtained. Of interest is that at this temperature the aqueous dispersion became transparent. The isotropic NMR signal suggests that small vesicles or micelles are predominant. The transition was reversible and the aggregated lamellar phase (as shown by NMR & turbidity) reappeared after incubation at room temperature.

^{31}P-NMR spectrum of acidified DPPC/TS (40/60) at 5°C indicated a mixture of lamellar and non-lamellar phases. At 20°C, the NMR spectrum showed a major component similar to that characteristic of a hexagonal phase (Fig. IV-1, b).

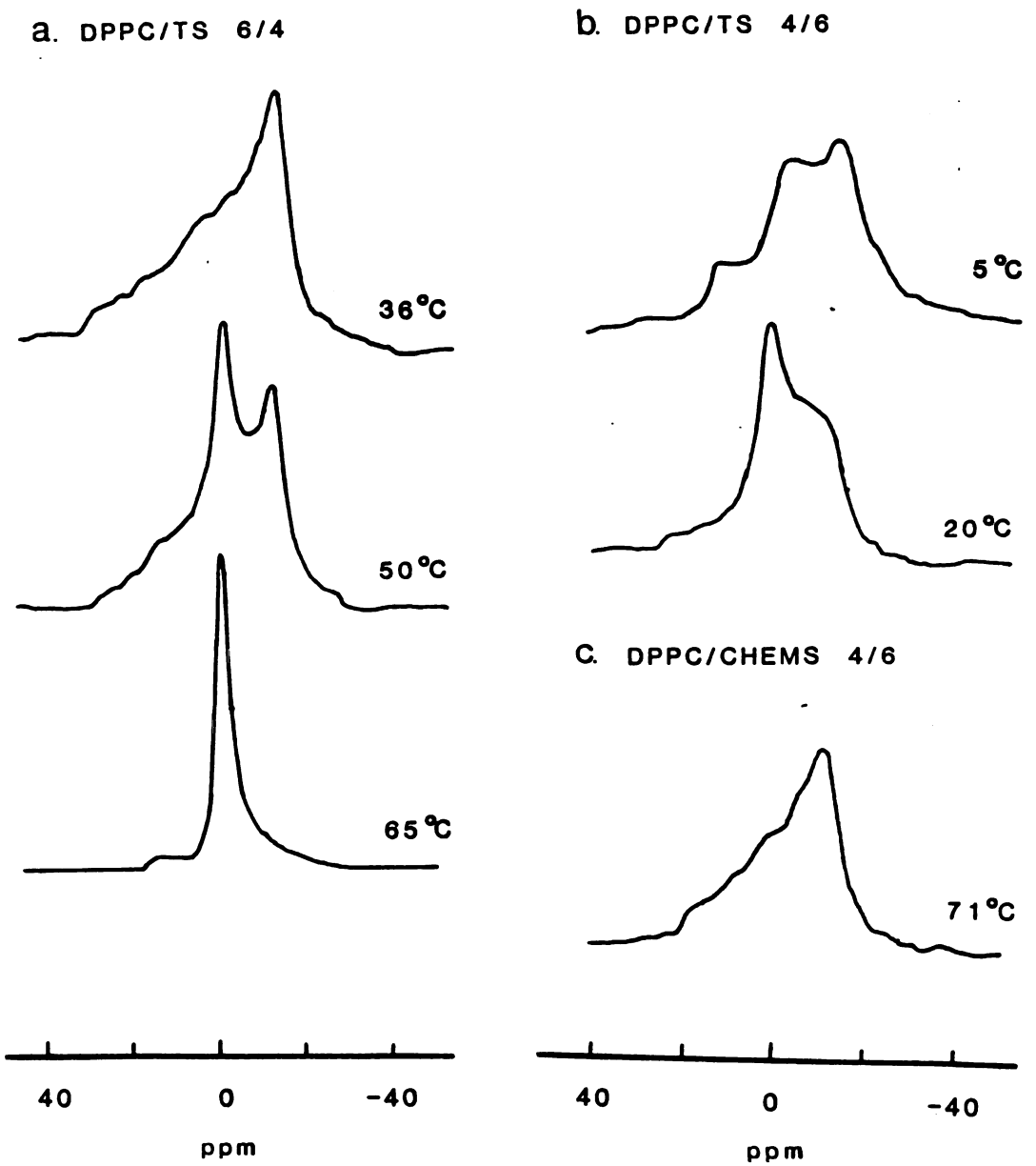
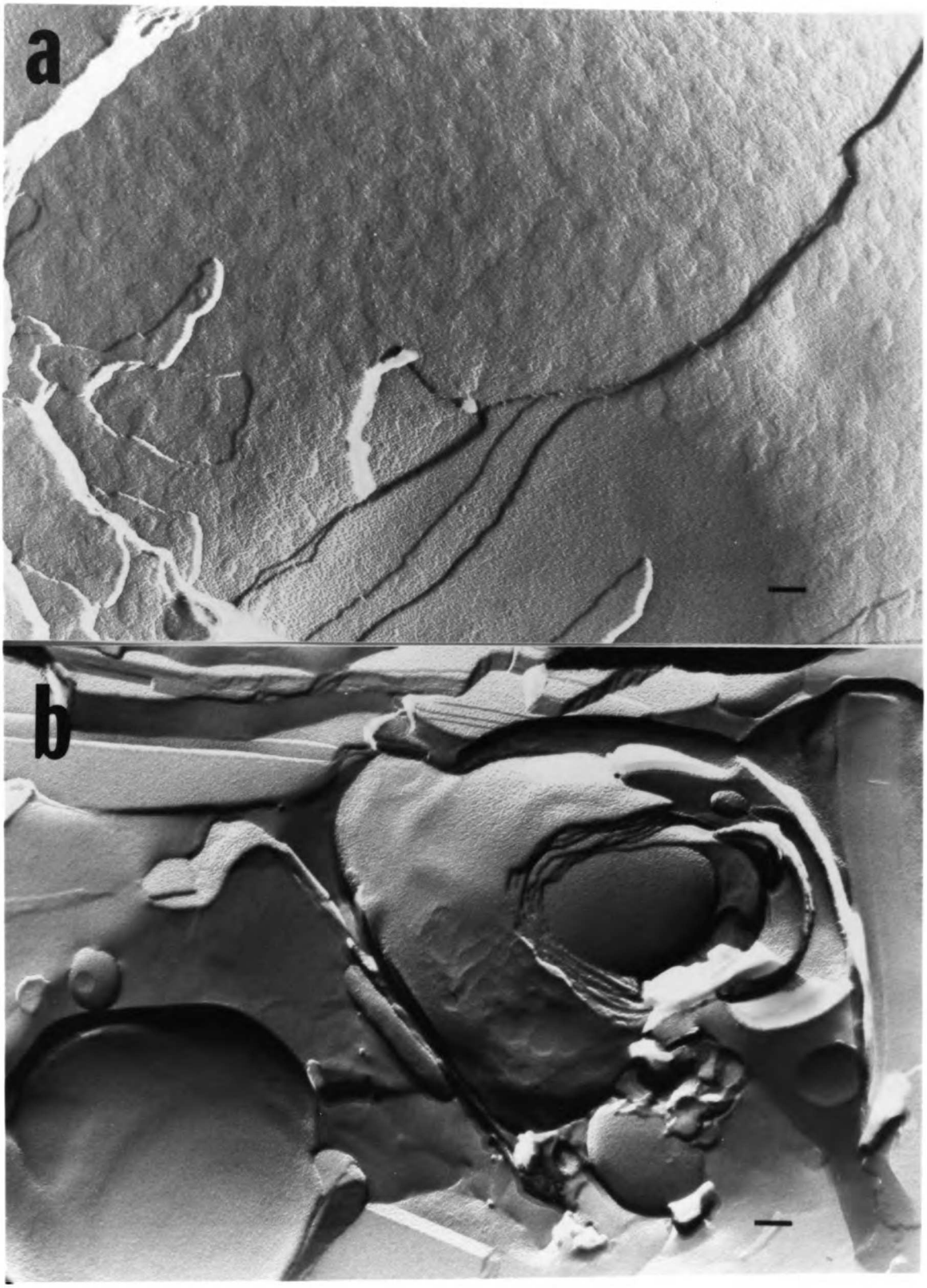


Figure IV-1. Proton-decoupled ^{31}P-NMR spectra at 40.5 MHz of acidified (a) DPPC/TS (60/40) at 36°C, 50°C and 65°C (b) DPPC/TS (40/60) at 5°C and 20°C (c) DPPC/CHEMS (40/60) at 71°C.

Figure IV-2. Freeze-fracture electron micrography of lipids at pH 4.5. (a) DPPC/TS (60/40) at 22°C, no glycerol; (b) and (c) DPPC/TS (40/60) at 22°C, no glycerol; (d) DPPC/TS (40/60) at 22°C, with 30% v/v glycerol; Bar is 0.1 μm.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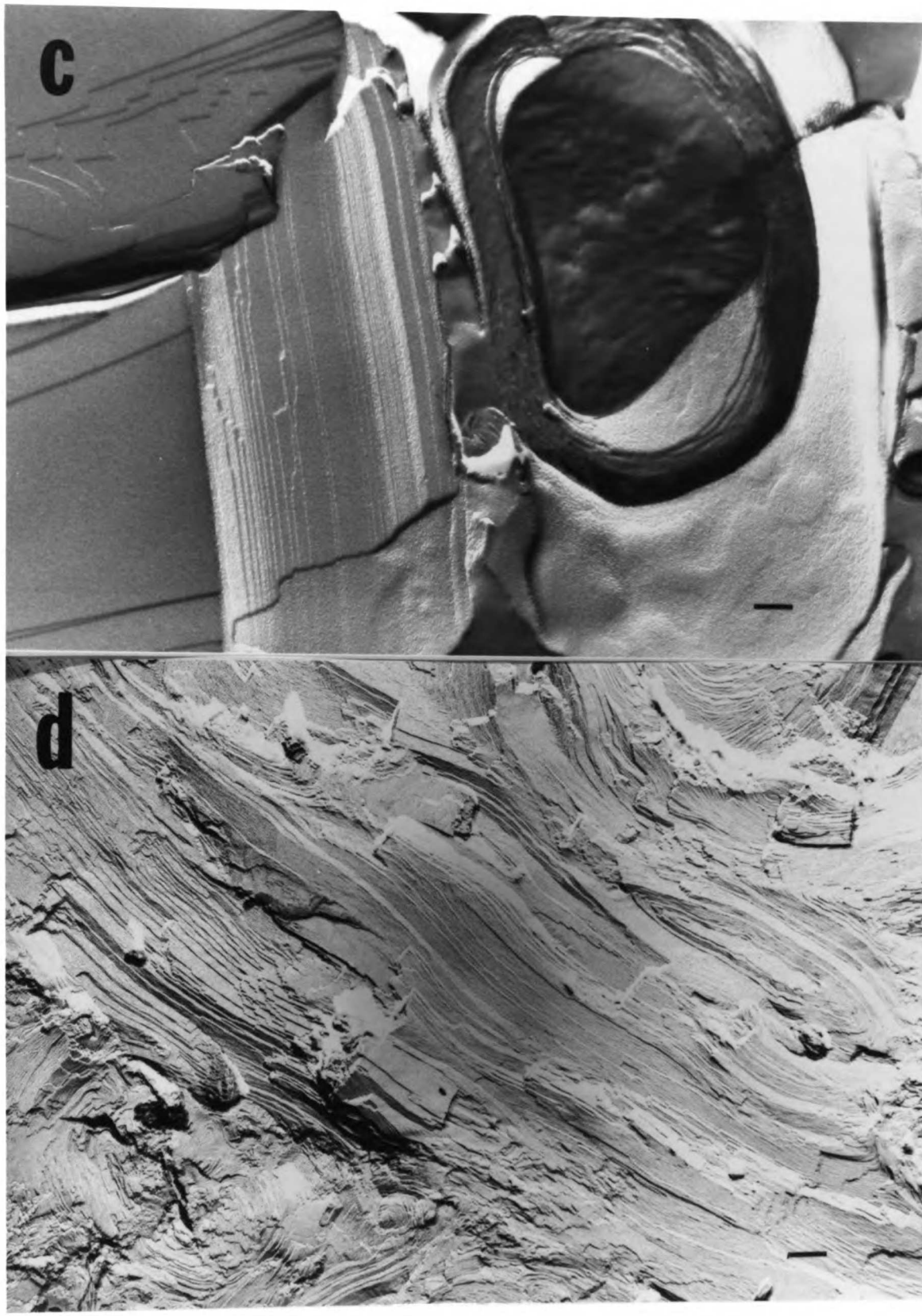
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d

X-ray diffraction and freeze-fracture electron microscopy of specimens rapidly frozen in the absence of cryoprotectants, however, did not reveal the presence of any hexagonal structures. Freeze-fracture of the lipid mixture rapidly quenched from 25°C showed two different types of structure: (1) aggregated vesicles with some bilayer irregularities (ridges) in the fracture faces (Fig. IV-2, b), (2) stacked lamellar sheets with very little aqueous spacing between them (Fig. IV-2, c). The cross-lamellar fracture showed straight edges with step breakings, suggestive of a two dimensional rigid lattice. X-ray diffraction gave two different lamellar repeat spacings, 80 Å and 60 Å, which may correspond to the multilamellar vesicular structures and the lamellar sheets observed by freeze-fracture electron microscopy. It may be noted that the inclusion of 30% glycerol to this lipid mixture prior to freezing resulted in a predominant structure very similar to hexagonal lipid cylinders (Fig. IV-2, d). Such a structure was not observed in the samples frozen without glycerol. This observation is similar to those of Bearer et al. (1982) which documented that the concentration of glycerol used as a cryoprotectant in freeze-fracture studies promotes the formation of lipidic particles. In Chapter III it has been shown that 30 % v/v glycerol reduced the L - H transition temperature of TPE-CHEMS mixtures by 10°C. This can be attributed to the dehydration effect of glycerol.

The addition of TS to DPPC broadens and shifts the gel-fluid transition of DPPC to a lower temperature (Chapter II). DPPC-TS of 4/6 composition at pH 7.4 has a asymmetric transition with the maximum of the endothermic peak at 21°C (Fig. IV-3). The acidified lipid preparation had a broadened DSC profile only slightly different from that of pH 7.4 (Fig. IV-3). This implies that a macroscopic phase separation has not occurred as the DPPC-TS mixture was acidified. However a phase inhomogeneity has been proposed for DPPC-TS mixtures at neutral pH where domains rich in DPPC and domains rich in TS coexist in bilayers (Chapter II). The detection of two separated phases in acidified DPPC/TS (40/60) by EM and X-ray diffraction may be the consequence of domain segregation.

5. Discussion

5.1. Lamellar Sheets

The formation of lamellar sheets has been suggested to be the preferred structure for amphiphiles with a head-group area close to the cross-section of the hydrophobic region (Israelachvili et al., 1980). Molecules with such a geometry cannot be accommodated in the curved surface of bilayers where flexibility is required. Several lipids with a small head-group and saturated acyl chains have been shown to exist in planar bilayer structures. The aqueous disper-

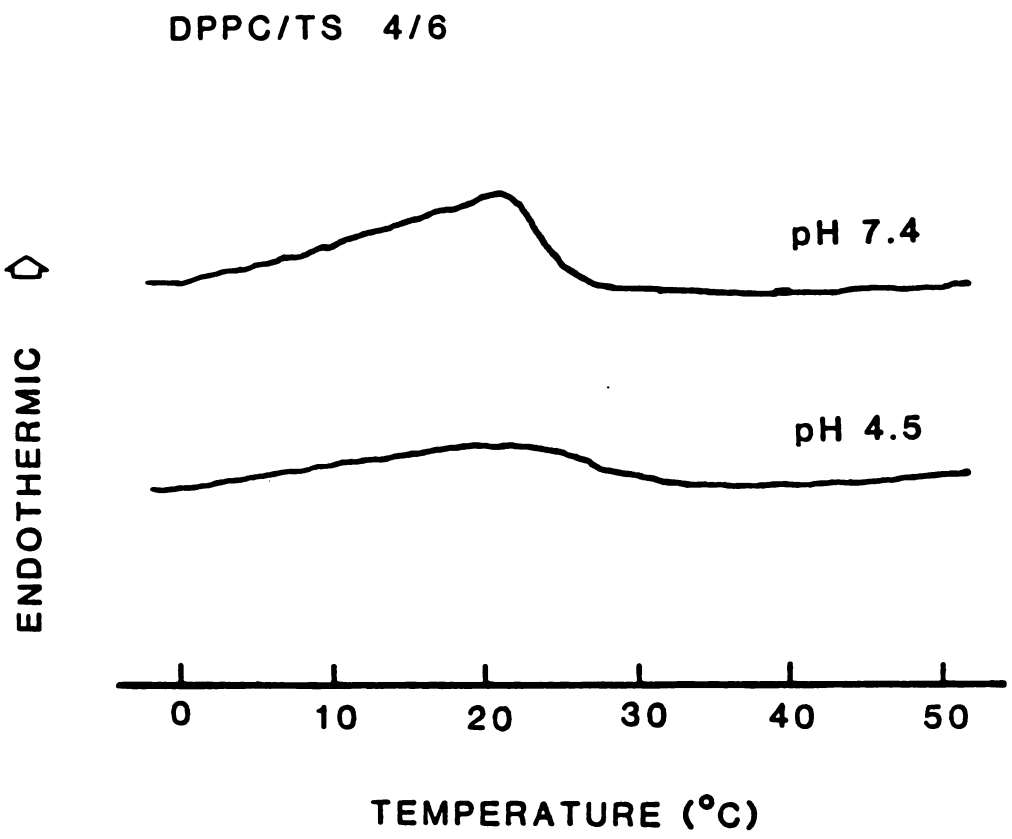

Figure IV-3. Differential scanning calorimetry of DPPC/TS (40/60) at pH 7.4 and 4.5. DSC was operated at a sensitivity of 1 mcal/sec and a scanning rate of 5°C/min.

sions of distearoylmonogalactosyldiglycerol form flat lamellar sheets (Quinn and Williams, 1983). The observation that dipalmitoylphosphatidylethanolamine cannot form intact vesicles at temperatures above the main transition (Singer, 1981) is probably because DPPE has such a molecular shape. In the present study the protonation of TS at low pH transforms DPPC-TS membranes into mixtures containing lamellar sheets. Since TS in the charged state has no effect on the bilayer structure of DPPC, the formation of planar bilayers under acidic conditions indicates that the protonated TS expands the hydrophobic region of DPPC into a matrix that is comparable to the phosphorylcholine headgroup. Such an effect is dependent on the content of TS, since DPPC with 40 mol% TS at pH 4.5 stay in a bilayer structure at 25°C. In addition, acidic amphiphiles with a similar pKa as TS but with a non-polar region smaller than that of TS are ineffective in destabilizing the bilayer structure. The addition of 60 mol% cholesterylhemisuccinate does not affect the bilayer structure of DPPC at pH 4.5 and 71°C as indicated by ^{31}P-NMR (Fig. IV-1, c). The acid-sensitive transformation of DPPC-TS we observe has been implicated in the DPPC-palmitic acid system reported by Marsh and Seddon (1982). However the apparent pKa of a fatty acid in phosphatidylcholine bilayers is relatively high (Kantor and Prestegard, 1978; Ptak et al., 1980; Schullery et al., 1981) so that a bilayer to non-bilayer transition occurs between pH 7.0 and 10.0 in the PC-fatty acid

system.

5.2. Generation of ^{31}P-NMR Spectrum of Hexagonal type

^{31}P-NMR spectrum of the hexagonal and isotropic types can be generated by changing the phosphate head-group conformation while retaining molecules in a bilayer structure (Thayer and Kohler, 1981; Noggle et al., 1982). Therefore the use of ^{31}P-NMR to study lipid polymorphism requires that the head-group phosphate is not altered in the transition between different phases (Seelig, 1978). Although this is true for most of the phospholipid systems, there has been instances where the lipid structure inferred from the ^{31}P-NMR line shape can not be confirmed by other techniques. Hui et al. (1980, 1981a) reported that electron microscopy and X-ray diffraction failed to detect the hexagonal structure suggested by ^{31}P-NMR on sphingomyelin and soybean PE/egg PC mixture. Bilayers of phosphatidyldiacylglycerol and phosphatidylcholesterol also generate ^{31}P-NMR spectra characteristic of both hexagonal and isotropic phases (Noggle et al., 1982). In the latter case, the two hydrophobic parts of the phospholipids are buried in the acyl chain part of the bilayer. Lipids with such a "horseshoe" packing may have a different orientation of the phosphate tensor and, consequently, a ^{31}P-NMR spectrum that might be similar to that arising from lipids in a H_{II} phase. We have shown here that DPPC containing protonated TS yields a ^{31}P-NMR spectrum indicative of a hexagonal phase, while X-ray diffraction and

electron microscopy indicate structures of bilayers and lamellar sheets. The bulky isoprenoid ring and branched phytanyl chain of tocopherol interact poorly with the saturated acyl chains of phospholipid (Maggio et al., 1977). A high content (25 mol%) of tocopherol markedly destabilizes egg lecithin membranes (Cushley and Forrest, 1977). Attachment of the succinyl group confers sufficient hydrophilicity on tocopherol so that it can remain in a bilayer with little effect on the phospholipid. The incorporated TS will destabilize phosphatidylcholine membranes immediately upon acidification to pH 4.5 at which the hydrophilicity of TS is lost. Under these conditions a high concentration of TS may have an effect on the head-group conformation of DPPC. Although further studies are required to elucidate the exact configuration of DPPC in the presence of protonated TS, it is clear that, in this system, a ^{31}P-NMR shape that has been considered to be diagnostic for the hexagonal phase is not associated with a hexagonal structure as observed by freeze-fracture EM or X-ray diffraction.

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