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STUDIES OF LIPID PHASE TRANSITIONS IN SOME MODEL MEMBRANES USING DIFFERENTIAL SCANNING CALORIMETRY

by

David J. Vaughan

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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March, 1976

St. John's Newfoundland
ABSTRACT

Phosphatidylcholines (PC's) and phosphatidylethanolamines (PE's) have been shown, in general, to comprise the major lipids of most biological membranes. Therefore, a systematic study using differential scanning calorimetry of DPPC, DPPE and their N-methylated intermediates as well as their diether analogues was undertaken in order to characterize the phase behaviour of such model systems with a view to understanding the more complex behaviour observed in real membranes.

It was observed that the lipid phase transition temperature (Tc) decreased as methylation of the PE head group increased. The transition enthalpies for these systems of single lipids showed no systematic variation with methylation.

It has been noted that saturated lecithins (as well as DPPC) display a pretransition occurring several degrees before the main hydrocarbon chain transition on DSC thermograms. The introduction of "foreign" molecules (even ones of very similar structure) in lecithin bilayers disrupts the long range order in the gel phase and either modifies or abolishes the pretransitional endotherm, depending upon the concentration of the perturbing molecule. Certain water soluble additives have no effect on the pretransition up to 20 mole %. However, cooling thermograms of such systems display a minor exotherm occurring ~12°K below the main exotherm which is absent in pure lecithin systems.

The tricyclic antidepressant, desipramine, has been shown to affect lipid phase behaviour. When included at equimolar concentrations with
single lipids it lowers the gel-to-liquid crystalline phase transition temperature and generally increases the enthalpy of the phase change. The extent of lowering of the phase transition temperature was found to be dependent on the head group structure of the phospholipids. PE's were affected greater than PC's in both diester and diether series of lipids. Binary mixtures of lipids were also analyzed calorimetrically, and when desipramine was included in these mixtures so as to be equivalent on a molar basis with one of the lipids it was found to exhibit a preference for the lower melting component.
ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to Dr. Kevin Keough for his guidance and encouragement during the course of this study. I would also like to especially thank Donna Osborne for typing this thesis and Phil Hyam for technical assistance in the preparation of certain photographic material. Financial support in the form of a RODA Summer Studentship and assistance from Memorial University and the Medical Research Council of Canada is gratefully acknowledged.
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LIST OF ABBREVIATIONS

DPPE: Dipalmitoylphosphatidylethanolamine
MM-DPPE: N-methyl dipalmitoylphosphatidylethanolamine
DM-DPPE: N,N-dimethyl dipalmitoylphosphatidylethanolamine
DPPC: Dipalmitoylphosphatidylcholine
DHPE: Dihexadecylphosphatidylethanolamine
MM-DHPE: N-methyl dihexadecylphosphatidylethanolamine
DM-DHPE: N,N-dimethyl dihexadecylphosphatidylethanolamine
DHPC: Dihexadecylphosphatidylcholine
PMPC: 1-Palmitoyl-2-myristoylphosphatidylcholine
DOPC: Dioleoylphosphatidylcholine
DOPE: Dioleoylphosphatidylethanolamine
DPPA: Dipalmitoylphosphatidic acid
DPPG: Dipalmitoylphosphatidylglycerol
DMPC: Dimyristoylphosphatidylcholine
DMPE: Dimyristoylphosphatidylethanolamine
DSPC: Distearoylphosphatidylcholine
DLPC: Dilauroylphosphatidylcholine
DSPE: Distearoylphosphatidylethanolamine
DLPE: Dilauroylphosphatidylethanolamine
lyso PC: 1-Palmitoyl-sn-glycero-3-phosphorylcholine
PE: Phosphatidylethanolamine
PC: Phosphatidylcholine
PG: Phosphatidylglycerol
DTA: Differential thermal analysis
<table>
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<tr>
<td>DSC</td>
<td>Differential scanning calorimeter (calorimeter)</td>
</tr>
<tr>
<td>Tc</td>
<td>Gel to liquid crystalline phase transition temperature</td>
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<tr>
<td>P.c.</td>
<td>Liquid crystal(line)</td>
</tr>
<tr>
<td>ANSA</td>
<td>1-Amino-2-naphthol-4-sulfonic acid</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenalin</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-Tetramethylpiperidine 1-oxyl</td>
</tr>
<tr>
<td>Kcal</td>
<td>Kilocalories</td>
</tr>
<tr>
<td>Sn</td>
<td>Stereospecifically numbered</td>
</tr>
<tr>
<td>O.K</td>
<td>Degrees Kelvin</td>
</tr>
<tr>
<td>M.</td>
<td>Molar (moles per liter)</td>
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<tr>
<td>O.D.</td>
<td>Optical density</td>
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INTRODUCTION

It is now generally accepted that the basic structure of most biological membranes consists of a bilayer of phospholipids with protein molecules either ionically bound at the bilayer surface with little or no penetration of the bilayer or interdigitated partially or completely through the hydrophobic portion of the membrane (1, 2). Therefore a thorough understanding of membrane components at the molecular level may help elucidate the many complex processes that occur in biological systems. Furthermore, it has been shown that the phospholipid components of several membranes play an important role in the efficient functioning of certain operations in the membrane and in fact the actual physical state of the lipids may serve as modulators of such activities (3, 4, 5). Depending on the respective concentrations of lipid and water, various lipid phases may be encountered (25). It has been reported that the critical micelle concentration for several phospholipids is extremely low and that at higher lipid concentrations a variety of ordered phases have been observed (73). For aqueous dispersions of phospholipids over a biologically relevant temperature range the predominant structural form noted is a bilayer configuration of lipids arranged in an ordered lamellar phase (25, 73, 76, 77) which may be more or less rigid (i.e., gel and l.c. phases) depending on external influences. Pure lipids and mixtures of lipids can be dispersed in an aqueous environment to take up a bilayer configuration and thus provide useful and simple model systems for the study of the intrinsic structure and function of biological membranes. In the bi-
layer configuration the lipids form a lamellar phase in which some of
the excess water is sandwiched between adjacent bilayers with the
rest of the bulk water completely free of the lipid layers (6). De-
pending upon the lipid, a variable amount of water may be "bound" to
lipid polar head groups so that it becomes unfreezable (6; 23).
Individually the lipids are oriented with the polar head groups
facing the aqueous phase and their non-polar hydrocarbon chains
pointing towards the center of the membrane. When a pure phospholipid
is heated from room temperature up to its capillary melting point a
number of thermotropic phase changes occur (i.e., phase changes caused by the effect of heat). This was first shown by infrared spectroscopy and later by thermal analysis and other physical techniques (7, 8, 9, 10). Similarly, in the presence of water, the phospholipids do not pass directly from a particular crystalline state to a solution but instead they exist in various hydrated phases (9). Generally speaking each phase obtained is a function both of water content and of temperature. The two lipid phases of concern to this study are the gel and liquid crystalline and the temperature at which a given lipid undergoes a gel to liquid crystalline (gel to l.c.) phase change is denoted as the transition temperature. At the present time there are no universally accepted designations for the gel and l.c. phases of hydrated lipids, e.g., both α- and β-(β'-) have been used to designate the gel form of hydrated DPPC in x-ray diffraction studies (11). Therefore, for the purpose of this study, no specific designation will be made for the gel phase or the l.c. phase.

Several changes occur as a lipid-water system is converted from a gel to a l.c. phase and they may be summarized as follows:

1. Expansion of molecular lattice and decrease in the thickness of the lipid bilayer.
2. Increased rotational isomerism of \(-\text{CH}_2-\) groups about C-C bonds.
3. Increased mobility of \(N(\text{CH}_3)_3\) groups (PC's).
4. Increased rate of diffusion of lipids above gel to l.c. transition temperature.
(5) Some change in bound water interactions at the transition temperature.

(6) Permeability of various molecules is generally greater with lipids in the l.c. state.

It has become apparent that many membrane phenomena such as transport processes and the activity of certain membrane-bound enzymes were associated with the actual physical state of the lipids in the membrane (3, 4, 5). For example, an enzyme may be active only when the associated lipids are in the l.c. phase or perhaps the activation may be modulated by a change in lipid phase. It has been shown (3) that a purified ATPase from lamb kidney outer medulla undergoes a large change in activation energy at 20° C as observed on an Arrhenius plot. It was also shown using a spin label probe, methyl 6-(\(\text{N-methyloxazolidinyl-N-oxyl}\))heptadecanoate, that the membrane lipids of the ATPase undergo a phase change at the same temperature (3). Other investigators have shown that the transport of drugs in Staphylococcus aureus, e.g., chlortetracycline, is related to membrane phase transitions (5). The bacteria S. aureus can be cultured in the presence of different fatty acids. Bacteria incorporating larger amounts of unsaturated and branched chain fatty acids had lower membrane transition temperatures than control cells. These transitions concurred with the temperatures obtained using Arrhenius plots and fluorescence techniques indicating that the mobility of chlortetracycline through the membrane was temperature, i.e., lipid phase, dependent.

DETECTION OF LIPID PHASE-CHANGES

Some of the physical methods used to detect lipid phase changes
include:

(1) Dilatometry

When lipids undergo a gel to l.c. phase transition, there is an expansion of the molecular lattice accompanied by a decrease in phospholipid bilayer thickness. The overall result of these changes is a volume expansion of the bilayer (12, 13). This volume change at the phase transition can be measured using dilatometry.

(2) Fluorescent probes

Certain probe molecules, e.g., 8-anilinonaphthalene-1-sulfonate (ANS) or its derivative, N-phenyl-1-naphthylamine (NAP), can interact with phospholipids at either the head group or hydrocarbon regions. These fluorescent dyes can monitor lipid phase changes since they show an increase in quantum yield and/or fluorescence intensity over the transition range (13, 14).

(3) Light scattering

The relative 90° light scattering intensity of many phospholipid dispersions changes very little with temperature until the phase transition temperature is reached. At that point there is a relatively sharp drop in scattering intensity along with a change in the refractive index of the system resulting in an increase in optical density. By monitoring O.D. changes with temperature the gel to l.c. lipid phase change may be observed. Phase changes in dispersions of DPPC (14) as well as oleic (cis 18:1), elaidic (trans 18:1) and palmitoleic (16:1) phospholipids (13) from fatty acid auxotrophs have been analyzed by light scattering.

(4) Spin label techniques

The electron spin resonance label TEMPO (2,2,6,6-tetramethyl-
peperidine 1-oxyl) exhibits different membrane solubilities depending upon the phase of the lipids in the membrane (15). Lipids at temperatures below their gel to l.c. transition temperature are in a more rigid state than lipids which are in the liquid crystal or more fluid state. The solubility of TEMPO in membranes is greatly increased when the lipids are in this more fluid phase (16). This being the case, spin label techniques can be used to detect phase changes in artificial bilayers and biological membranes by monitoring spin label solubility (as observed on the first derivative esr spectrum) as a function of temperature. Probes that are attached to fatty acids have also been used but not as successfully as the TEMPO-like probes. It has been shown that the stearic acid spin label probe tends to migrate to the more fluid lipid phase and may not provide an accurate picture of membrane fluidity or rigidity (17, 18).

It is worth noting that esr techniques as well as fluorescence measurements have a special usefulness because of the small amounts of material needed. Such an advantage has made these techniques popular for biological membranes where sufficient material for study is sometimes difficult to obtain.

(5) Nuclear magnetic resonance (nmr)

Early wide-line studies suggested that nmr was a promising technique with which to study molecular mobility in lipid systems (19). When lipids undergo a gel to l.c. phase change the head group and chain mobility is greatly increased yielding nmr spectra characteristic of this increased motion (20). Additional information on lipid phase changes may also be obtained using high resolution nmr (21). High resolution proton nmr may be of limited usefulness for the study of
membranes (21), but \(^{13}\text{C}\) and \(^{31}\text{P} \) nmr may yet provide substantial information. The ability for specific enrichment of lipids with \(^{13}\text{C}\) would make it a very powerful tool.

(6) X-ray diffraction

X-ray diffraction studies have been performed on a variety of lipid-water systems yielding information on their general properties as well as a classification of lipid structures (25). Recent x-ray studies on DPPC (26) have shown the existence of three distinct lamellar phases. These phases are temperature dependent and as such reflect the state of the lipid, i.e., gel or l.c. Crystallographic analysis of these lamellar phases led to the measurement of the specific x-ray spacings for each phase. It was therefore possible to characterize each of the phases and measure a temperature for the gel to l.c. transition. X-ray diffraction studies of natural membranes have also been performed on both multi-membranes (27) and single membranes (28, 29).

(7) Calorimetry

There are two main calorimetric methods available to monitor the gel to l.c. phase transition of lipids (23), differential thermal analysis (DTA) and differential scanning calorimetry (DSC). In DTA the sample and an inert reference material are heated or cooled at the same rate and the difference in temperature between them is recorded. The differential temperature remains zero or constant until a phase change occurs in the sample when the differential temperature increases until the transition is completed and then decreases again. This differential temperature is amplified and outputted on a strip
chart recorder. Thus a peak corresponding to the lipid phase change is observed.

The major technique used to detect lipid phase transitions in this study was differential scanning calorimetry (DSC) and a more thorough description of this method follows.

In DSC the sample material and the reference are heated independently so that their temperatures are at all times equal or in a constant relation to one another. When a gel to l.c. transition or phase change occurs heat is absorbed or evolved by the sample and therefore more or less energy is required to maintain the sample temperature with respect to that of the reference. During a phase change it is this differential power that is measured.

The analyzing unit of the DSC consists of two holders, one for sample and one for reference, encased in a large aluminum block acting as the heat sink which can be maintained at a convenient temperature. Since the calorimeter is not very responsive at temperatures near the block temperature, it is necessary to maintain the block at temperatures at least 25° lower than the anticipated transition temperature. In many cases, for samples of biological interest, it is convenient to maintain the block temperature at the boiling point of liquid nitrogen, i.e., 77°K. Cooling is achieved by letting the sample cool freely. Programmed cooling rates may be carried out by limiting the rate at which heat is lost from the sample holder to the surroundings by applying power to the analyzing head. The analyzing unit atmosphere is dynamic with inert purge gases nitrogen and helium flowing through the sample chamber continuously. Volatile samples are sealed inside
aluminum pans.

Phospholipid samples are dispersed in water and then placed in aluminum sample pans which are then sealed. Water is an essential component of the artificial system and to avoid loss through evaporation the pans must be sealed. The capacity of the sample pans is 20 μl but normally approximately 10 mg of phospholipid dispersion is analyzed per pan. Too large a sample size results in broken pans either by freezing damage or high vapor pressure breaking the seal.

The technique of DSC was chosen for studying phase transitions because it avoids the use of probe molecules which may perturb the system. Certain esr probes, e.g., probes attached to fatty acids, were prone to giving a false picture of membrane fluidity. Apparently probes of this type were likely to solubilize in the more fluid membrane regions even if such regions constituted only a small percentage of the overall membrane (17, 18). It has been found since that other probes, e.g., TEMPO (15) and the fluorescent probe parinaric acid (22), yield more reliable results. The disadvantage of DSC lies in its low sensitivity compared with other physical techniques.

Samples for calorimetric analysis are usually in the mM range while the concentration of esr probes, for example, is in nanomoles.

INFORMATION AVAILABLE FROM DSC ON LIPID PHASE CHANGES

Fig. 1 shows the usual thermotropic phase transitions observed with aqueous dispersions of many lipids as obtained by differential scanning calorimetry. Both transitions are endothermic indicating that heat is absorbed by the sample. The lower endotherm is the
ice to water phase change while the upper melt is the gel to l.c. transition of the lipid. The water endotherm in phospholipid dispersions is not observed calorimetrically until the concentration of water exceeds approximately 20 mole % (23). Water present at lower concentrations is bound by the lipid about its polar head group region and shows no classical freezing or melting behavior. Lipids, therefore, are generally dispersed in excess water (lipid:water, 1:1 or 1:2 w/w) and analyzed on the DSC starting at below zero temperatures. Large excesses of water (e.g., 100:1) although more like a true membrane system can't be conveniently analyzed on the calorimeter because of baseline drifting and sensitivity problems. The observation of the ice to water phase change ensures that sufficient excess water is present such that the lipids are forming lamellar phase bilayers consistent with biological membranes (23).

The transition temperature may be defined in two ways. For highly cooperative transitions and for sharp phase changes involving standards, the transition temperature is obtained by drawing a tangent to the slope of the phase change where it departs from the baseline. The intersection of this tangent with the baseline is defined as the transition temperature or Tc (24). Alternatively this temperature may be defined as the peak of the transition, the point where the sample is most rapidly absorbing heat. This temperature is defined as Tm. Both temperatures are widely used but for this study the Tc definition of transition temperature has been adopted. Tm gives the point of most rapid change. The area under the lipid curve is directly proportional to the enthalpy of transition and the melting

* See Appendix I.
range of the phase change is an index of the cooperativity of the transition.

ARTIFICIAL LIPID BILAYERS AS MODELS FOR BIOLOGICAL MEMBRANES

Recent work has indicated that much of the lipid in biological membranes exists in the form of a bilayer. Therefore a systematic analysis of the phase behaviour of artificial membranes of pure lipid bilayers in water would provide a basis for understanding the more complex nature of real membranes.

Phase transitions in biological membranes may play a vital role in many membrane processes since such processes have been shown to be related to the particular state of the surrounding lipid. For example, the transport of sugars or drugs across membranes or the activities of many membrane enzymes are enhanced when the associated lipids are in the more fluid l.c. phase (3, 4, 5). Arrhenius plots for some membrane-associated enzymes show discontinuities at temperatures closely aligned with the gel to l.c. phase transition of the lipids associated with the enzyme (3, 4, 5). Compounds, therefore, that can interact with lipids and change their physical state may serve as modulators of such membrane processes.

Many compounds are known to interact with lipids and modify their phase behaviour, i.e., Tc and/or enthalpy values. Among these are several proteins (30) and steroids (23, 31), metal ions (32), foreign molecules in general (33) and drug molecules (34). Included among the latter is the tricyclic antidepressant drug, desipramine.
The phase behaviour of disaturated phosphatidylcholines in water is unusual in that two transitions are observed calorimetrically - a major transition associated with a fluidization of the hydrocarbon chains and a minor or pretransition which occurs some 2 - 7°C below the main chain "melt" depending on fatty acid chain lengths. The major transition is associated with an increased molecular motion of the hydrocarbon chains is going from the rigid gel state to the less rigid (but still partially ordered) l.c. phase.

The origin of the pretransition is not fully understood and several possible explanations are available. Studies using nmr (19) have shown that just prior to the main chain melt in DPPC the mobility of the head group is increased implying that this increased motion could be the source of the pretransition. There is also the possibility of a reorganization of the water at the lipid/water interface such that a head group-water reorientation is effected. Alternatively the source of the pretransition may be the chains and not the head group region. In this regard we may consider a reorganization of the hydrocarbon chains either through cooperative rotation along the axes or through a tilting of the hydrocarbon chains with respect to the plane of the bilayer. Recent x-ray and monolayer studies (26) strongly support the tilting of the hydrocarbon chain in the gel phase as being the source of the pretransition.

Whatever the origin of the pretransition it is quite sensitive
to impurities and thus provides a simple way to investigate the effect of small perturbations on long range order in lipid bilayers. It has been observed previously that 10 mole% DMPE in DMPC bilayers abolishes the pretransition but that the pretransition is evident in bilayers of all proportions of DMPC and DPPC (33). With respect to biological systems it has been found that mammalian lung surfactant is relatively rich in DPPC (59). Since the pretransition of DPPC occurs at biologically relevant temperatures (345–37.5°C), it is of interest to study the effects of certain molecules on the pretransition.

**PHASE SEPARATION IN LIPID-WATER SYSTEMS**

Since biological membranes contain a wide variety of lipids the question arises as to whether or not these lipids are homogeneously distributed throughout or reside in heterogeneous packets within a membrane. In other words is there a random distribution of the lipids in a membrane bilayer or do the lipids segregate forming clusters of one type or another depending on composition or fluidity.

The first studies on phase separation of lipid-water systems were done on binary mixtures of lecithins using calorimetry (23). Mixtures of DSCP-DPPC and DSCP-DMPC were analyzed and the phase diagram of the DSCP-DPPC mix showed that below the Tc line a series of solid solutions were formed. It was concluded that compound formation did not occur and with this pair of molecules having only a small difference in chain length co-crystallization occurred. With the DSCP-DMPC mixture the difference in chain length was too great for co-crystallization to occur and as the system was cooled migration of
lecithin molecules ocurred within the bilayer to give crystalline 
regions corresponding to the two components. Since that time many 
examples of lipid heterogeneity both in real and artificial systems 
have been examined.

The spin label probe TEMPO, for example, has been used to in-
vestigate phase separation in binary mixtures of various lipids and 
in fact the phase diagrams for these systems could be obtained (35, 
36). These phase diagrams showed that for binary mixtures of PC's 
that have acyl chain lengths differing by more than two carbons or 
for DPPC-DPPE, DPPC-BMPE mixtures an apparent discontinuity in slope 
is observed indicating a limited gel phase miscibility. More recently 
(36), it has been reported that phase separation of lipids can occur 
when the lipids are in the fluid l.c. phase. This immiscibility could 
give a lateral phase separation into fluid domains in the plane of 
the membrane, and/or a transverse phase separation into an assymetrical 
bilayer membrane, and/or possibly discontinuous bilayer membranes of 
different composition. Other workers have shown that lipid phase separation 
can occur in phosphatidic acid-lecithin membranes due to the effects of 
calcium ions (37).

It was originally thought that membrane lipids existed in only the 
fluid state. Recently, however, it has become apparent that certain mem-
branes, e.g., Acholeplasma laidlawii B., can exist with lipids in both 
the l.c. and gel state (68). This would imply a certain degree of 
segregation among the membrane lipids resulting in a form of phase 
separation. It is conceivable that the lipids separate on the basis
of class or fluidity resulting in not only lateral but also trans-
bilayer asymmetry. It has been shown (38) that cosorption of
equimolar quantities of PG and PC results in bilayered vesicles,
the outer surface of which contains on the average twice as many
PG as PC molecules. The activity of certain proteins in membranes has
been shown to be dependent on the nature of the surrounding lipid (3, 4)
and therefore compounds affecting the phase of these lipids could be
of biological importance. Transmembrane asymmetry of lipids has also
been reported in erythrocytes (40, 62).

It is noted that most physical techniques may not be able to
distinguish unequivocally between separate gel and l.c. phases in the
same bilayer and separate bilayers of gel and l.c., even though most
authors have interpreted these results to indicate the first al-
ternative. Our results indicating phase separation are subject to
this same cautionary note.

PURPOSE OF STUDY

It has been shown that PC's and PE's in general comprise the
major lipids of most membranes but only recently has information be-
come available on the phase behaviour of these compounds in model
membranes. With saturated phospholipids in water it was observed that
the gel to l.c. phase transition temperatures of PE's are substantially
higher than those for corresponding PC's and that the saturated
lecithins display a small endothermic pretransition a few degrees be-
low the main chain transition which is absent in PE's (23, 32, 33, 35, 39,
50, 59). Therefore, a study of the phase properties of artificial
systems using DSC would be useful as a basis for further work.

The pretransition observed with disaturated lecithins has also been investigated with a view to determining how small a difference in structure a foreign molecule must have in order to abolish the pretransition and at what concentration of perturbing molecules is the disappearance of the pretransition complete. A series of experiments were performed in which structurally similar compounds were added to DPPC to see if the pretransition was affected. Analogous experiments were performed with DHPC systems. It has recently been shown that desipramine is capable of affecting the phase characteristics of model lecithin bilayers (34). With this in mind, it was decided to extend these investigations to a complete series of related lipids to study what effects this drug molecule has on their phase behaviour.

Mixed lipid systems more closely approximate a biological membrane and therefore studies on mixtures of PE's and PC's have been undertaken. A series of experiments using desipramine with mixed lipid systems were performed to see if the drug exhibited any selective interaction with one of the lipid components. Such observations might be extended to biological membranes with a view to explaining the possible mode of action of desipramine.
MATERIALS AND METHODS

Chemicals

All lipids were purchased from Calbiochem, La Jolla, California, with the following exceptions: PMPC was a product of Supelco Inc., Bellefonte, Penn.; DOPC, DOPE and DPPA were purchased from Serdary Research Laboratories, London, Ontario; Dipalmitin (purum) was obtained from Fluka Buchs; Lyso PG was synthesized in this laboratory. Perchloric acid, 1-amino-2-naphthol-4-sulfonic acid (ANSA), ammonium molybdate, calcium chloride, NaCl, Hyflo Super Gel and reagent grade solvents were obtained from Fisher Scientific, Montreal, Quebec. Silica Gel G was a product of Merck, Germany and Silica Gel N-HR was a product of Macherey, Nagel and Co., Düren, Germany. Silicic acid (100 mesh) was obtained from Fluka Buchs. Choline chloride, acetylcholine chloride and ethanolamine were purchased from Sigma Chemical Company, Montreal, Quebec. D-Mesipramine hydrochloride* (10,11-Dihydro-5-[3-(methylamino)propyl]-5H-dibenz[b,f]azepine) was the kind gift of Ciba Geigy, Canada Ltd., Dorval, Quebec. Grotalus Adamanteus venom was obtained from the Miami Serpentarium, Miami, Florida.

All the lipids except dipalmitin were found to be pure when checked by thin layer chromatography in solvent systems for neutral and phospholipids. Dipalmitin was found to contain principally the 1,2-diglyceride with a very small amount of the 1,3-diglyceride. All other chemicals were of the highest purity commercially available.

* See Appendix II.
Thin Layer Chromatography (TLC)

Silica Gel N Plates - Phospholipids

Phospholipid N-plates were made from Silica Gel N-HR. A slurry of 30 gms gel in 60 - 70 ml water provided enough material to cover five plates to a thickness of 1 mm. The plates were then dried at room temperature and stored. Before use the plates were activated at 110° C for 30 - 60 minutes. Phospholipid samples (1 mg) in a convenient solvent were applied via a microliter syringe. The plates were developed in various solvents based on systems designed for phospholipids (64,63).

Solvents: by volume

\[ \text{CHCl}_3: \text{CH}_3\text{OH}: \text{CH}_3\text{COOH}: \text{H}_2\text{O} \ (50:30:6:3) \]
\[ \text{CHCl}_3: \text{CH}_3\text{OH}: \text{NH}_4\text{OH} \ (63:30:5) \]

Detection of materials was carried out by staining the plates with iodine vapor. Alternatively the plates were sprayed with Dittmer-Lester reagent to detect phosphate esters (66). DPPE can also be identified by charring with \( \text{H}_2\text{SO}_4 \) or spraying with ninhydrin (66).

Silica Gel G Plates - Phospholipids/Neutral Lipids

G plates (containing \( \text{CaSO}_4 \)) were made by the same method as were N plates using the same gel/water ratios. Drying, activation and application of sample were the same in both cases. Detection was by the methods described for N plates. Solvents were based on systems designed for natural and phospholipids. (64, 65, 67).

Solvents: by volume

\[ \text{C}_6\text{H}_{14}:(\text{C}_3\text{H}_7)\text{O}_2: \text{CH}_3\text{COOH} \ (60:40:4) \]

* 1.5 M \( \text{NH}_4\text{OH} \)
Detection of Fatty Acids on G Plates

Plates were sprayed with \( \text{H}_2\text{SO}_4 \) 70% (v/v) saturated with \( \text{K}_2\text{Cr}_2\text{O}_7 \), then heated in an oven at 180°C for 30 minutes. Fatty acids appear as blackened spots.

Synthesis of Lyso PC—Based on the method of Wells and Hanahan (63)

DEPC (150 mg) was dissolved in a mixture of methanol (31 ml) and diethyl ether (9.6 ml). Phospholipase A (4 mg/mg) (Crotalus Adamanteus) was dissolved in 10 mM \( \text{CaCl}_2 \) and 300 \( \mu \)l of enzyme was used for every 150 mg lecithin. The lecithin-venom mixture was shaken for a few minutes, stoppered and incubated for three days at room temperature on a wrist-action shaker (63). The reaction is then stopped by the addition of absolute ethanol (3 ml). The organic solvents were blown off under a stream of nitrogen and the dry product was taken up in chloroform. The total lipid content is determined by the method of Fiske and Subbarow as modified by Dawson (60).

The chloroform solution containing lyso PC was chromatographed on a silicic acid:Hyflo Super Gel (1:1 w/w) column. Approximately 1 gram of silicic acid per mg phosphate were used. The column was then eluted with chloroform, chloroform-methanol (1:1 v/v) and finally methanol. Lyso PC eluted in the chloroform-methanol fraction and the concentration of lipid was determined again by the method of Fiske and Subbarow. All fractions were checked by TLC G plates using \( \text{CHCl}_3: \text{CH}_3\text{OH}: \text{H}_2\text{O} \) (65:24:4 v/v/v) as the eluting solvent.
Phosphate Determinations

(1) Fiske and Subbarow (as modified by Dawson) (60).

This method will effectively detect inorganic phosphate within a range of 2 - 40 μg/m phosphate. Phospholipids in organic solution must be dried under a stream of nitrogen and then digested for a few minutes or until the solution is clear with 0.7 ml HClO₄ (70%) on a hot plate to yield inorganic phosphate. Water (8.3 ml) is then added to each sample followed by the addition of 0.5 ml of 5% ammonium molybdate which forms a colorless or light yellow complex with the phosphate. This complex is then reduced yielding a blue color by the addition of 0.25% ANSA (0.5 ml) (1-amino-2-naphthol-4-sulfonic acid). The final volume is 10.0 ml. The optical density of the blue complex is read between 30 - 60 minutes after the addition of ANSA at 660 μm on a Unicam SP 500 spectrophotometer.

(2) Bartlett Phosphate Determination (61)

This method which is basically a modification of the Fiske and Subbarow determination is generally used for inorganic phosphate in the 0 - 2 μg/m range. 1.0 ml of HClO₄ (70%) is used to digest phospholipid samples on a hot plate for a few minutes or until the solution goes clear followed by the addition of 8.0 ml water. The ammonium molybdate (5%) and ANSA (0.25%) (0.5 ml each) are added and the resulting mixture (final volume 10.0 ml) is heated in a water bath (80 - 100° C) for 10 minutes to allow for maximum color development. A blue complex of the same type as in the Fiske and Subbarow is produced but since more acid is used the wavelength maxima is shifted to a higher value. The optical density was read
immediately at 800 μ on a Unicam SP 500 spectrophotometer.

**Determination of Tc in Mixed Lipid Systems**

In mixed lipid samples and lipid-drug dispersions where the melting behaviour is more complex, the usual method for determining Tc must be qualified. Where reasonably sharp phase changes are observed the method stated in the Introduction may be used to obtain the transition temperature. In cases where there is a broad uncooperative leading shoulder which merges into a more highly cooperative transition peak then the tangent is drawn to the slope of the more cooperative transition. The intersection of this tangent with the baseline then defines the Tc for the lipid mix or lipid-drug complex.

**Preparation of Samples for DSC**

1. **Pure Lipids**

   Lipid dispersions were made in deionized glass distilled water by heating the lipid-water (approximately 1/2, w/w) mixtures 10 - 15°C above the expected gel to l.c. transition temperature and dispersing thoroughly.

2. **Lipid Mixes**

   Lipids were mixed in chloroform/methanol (1:1 v/v) or chloroform alone and the solvent was removed under a stream of nitrogen. The samples were then placed in a vacuum for one hour to remove the last traces of solvent. In some cases, particularly with studies involving the lecithin pretransition, P₂O₅ was placed in the vacuum flask as an extra dessicant. The appearance of the pretransition is apparently

* See Appendix I.
affected by small quantities of solvent hence the added precaution with P₂O₅.

The dried lipid mixtures were then dispersed in deionized glass distilled water (lipid:water, approximately 1:2 w/v) at temperatures 10 - 15°C above the transition temperature of the higher melting component and mixed on a vortex mixture.

(3) Lipid Samples and Desipramine or Choline, Acetylcholine, Ethanolamine, NaCl

Desipramine was added to pure lipids or binary lipid mixtures either by intimately mixing the drug and lipids in organic solution followed by evaporation of the solvent and dispersal of the lipid/drug mix in water (mix:water, approximately 1:2 w/w), or by dispersing the dried single lipid or lipid mixture in an aqueous solution of desipramine.

Choline and acetylcholine were added to lipids by either of the above methods. Dried lipid samples were necessary for dispersal in aqueous solutions of ethanolamine and NaCl (165 mM).

In systems composed of single lipid species, desipramine was included at equimolar concentrations (lipid:drug, 1:1). In binary lipid mixtures, desipramine was included at a molar concentration, equivalent to either individual lipid component (lipid:lipid:drug, 1:1:1). The mole % of all other additives to lipid systems was variable and is stated for each case in the Results section.

After analysis on the DSC all samples were extracted from the aluminum pans by dissolving the lipid systems in chloroform-methanol (1:1 v/v) for phosphate analyses.
Calorimetry

Calorimetric analysis of lipid phase changes were performed on a Perkin-Elmer DSC-2 differential scanning calorimeter. Thermograms were usually obtained at a rate of $10^0$ K/min with a full scale sensitivity of 10 mcal/sec using air as reference. All samples were heated and cooled at least twice and reproducible thermograms were consistently obtained.

Standardization of Calorimeter

Calibration of temperature and areas for enthalpy determination were made using pure Indium, 99 mole-% benzene and 99.5% stearic acid as standards.

Determination of Areas

Areas of transitions on the thermograms were determined using a fixed arm planimeter. The area under such a curve is affected by (i) calorimeter sensitivity (ii) recorder chart speed (iii) sample size (iv) heating and cooling rates. Thermograms obtained under conditions different from those normally employed may be interpreted by taking into account the factors affecting peak size and shape. In the studies employed here, all samples were analyzed at the same heating and cooling rates, sensitivity and chart speed as were the standards.
RESULTS

SINGLE LIPID-WATER SYSTEMS

As previously mentioned phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) comprise the major lipids of many membranes. Therefore, a systematic study of the phase characteristics of artificial systems may help elucidate the more complex behaviour in biological membranes. In particular it was decided to investigate model DPPC and DPPE membranes and to note the effects of minor structural modifications on the phase characteristics of such systems so that these compounds could be used for subsequent investigations of the effects of perturbing agents.

Fig. 1 shows the heating thermogram tracings of water dispersions of dipalmitoyl PC, dipalmitoyl PE, dihexadecyl PC, dihexadecyl PE and the N-methylated derivatives of both the ester and ether series. The phosphatidylethanolamine derivatives exhibit the highest transition temperatures in each series. The addition of methyl groups to the polar head region tends to lower the phase transition temperature by an amount varying from 5.3 to 11.2°K. The diether analogues display consistently higher transition temperatures (2.8 - 4.6°K) than the corresponding diester lipids. Table 1 summarizes the transition temperature and enthalpy data associated with the gel to liquid crystal phase change of these two lipid series.

As stated, there would appear to be an inverse relationship between transition temperature, Tc, and head group methylation. In each series the PEs possess the highest Tc's with the PC derivatives having
Figure 1.

Tracings of DSC heating thermograms of aqueous dispersions of dipalmitoyl and dihexadecyl PC’s and PE’s and their N-methylated intermediates. The arrow here and in the other figures indicates heat flow into the sample, i.e., an endothermic change occurs in the direction of the arrow. Abbreviations listed in the text. The slope of the baseline in thermograms depends on both the amount and type of sample and on exact location of sample pans in the analyzing heads. In some but not all cases the slope can be compensated for by an instrumental adjustment. The determination of transition temperatures and enthalpies are not affected by sloping baseline as long as the slope is consistent.

(a) DPPE  (e) DM-DPPE
(b) DHPE  (f) DM-DHPE
(c) MM-DPPE  (g) DPPC
(d) MM-DHPE  (h) DHPC

The structures of these compounds are given in Appendix II. The low temperature endotherm is the ice to water phase change.
Table 1

Transition Temperature (Tc) and Enthalpy of Transition Data for Single Lipid Systems

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Transition Temperature Tc (°K)</th>
<th>Transition Temperature Changes (°K)</th>
<th>Enthalpy (kcal/mole Phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aDHPE (5)*</td>
<td>341.3 ± 1.5</td>
<td>-</td>
<td>7.64 ± 0.10†</td>
</tr>
<tr>
<td>MM-DHPE (5)</td>
<td>335.2 ± 1.3</td>
<td>6.1</td>
<td>9.09 ± 0.55</td>
</tr>
<tr>
<td>DM-DHPE (5)</td>
<td>324.0 ± 1.2</td>
<td>11.2</td>
<td>9.53 ± 0.44</td>
</tr>
<tr>
<td>DHPC (5)</td>
<td>316.6 ± 1.2</td>
<td>7.4</td>
<td>9.40 ± 0.54</td>
</tr>
<tr>
<td>bDPPE (7)</td>
<td>336.7 ± 1.2</td>
<td>-</td>
<td>8.79 ± 0.59</td>
</tr>
<tr>
<td>MM-DPPE (5)</td>
<td>331.4 ± 1.2</td>
<td>5.3</td>
<td>8.63 ± 0.53</td>
</tr>
<tr>
<td>DM-DPPE (6)</td>
<td>321.2 ± 0.7</td>
<td>10.2</td>
<td>10.04 ± 0.86</td>
</tr>
<tr>
<td>DPPC (4)</td>
<td>313.5 ± 0.5</td>
<td>7.7</td>
<td>8.50 ± 0.30</td>
</tr>
</tbody>
</table>

* Numbers in parentheses refer to number of determinations.
† Values reported as mean values ± 95% confidence limits.

a Diether lipid series.
b Diester lipid series.
c Tc of any given lipid subtracted from Tc of the previous lipid in the same series, e.g., Tc(DHPE) - Tc(MM-DHPE) = 6° K.
the lowest. The transition enthalpies do not show any systematic variation with methylation. In the diester series it appears that any methyl substitution on the DHPE head group raises the enthalpy to an approximately constant value ranging from 9.09 to 9.53 kcal/mole phosphate. In the diester series it seems that the addition of two methyl groups to the DPPE head group raises the enthalpy to a significantly higher value (p < .025) which is lowered by the subsequent addition of a third methyl group to the approximately equivalent range 8.50 to 8.79 kcal/mole phosphate obtained with the other three derivatives in the series.

THE EFFECT OF PERTURBING FOREIGN MOLECULES ON THE PRETRANSITION OF DPPC- AND DHPC-WATER DISPERSIONS

The understanding of phase behaviour in membranes and the effects of possible modifications by perturbing molecules is of interest with respect to a thorough understanding of order in biological membranes. We have attempted to analyze the effects of introducing perturbing molecules which cause minor changes and one compound which causes drastic changes in phase transition behaviour.

As has been noted previously the disaturated lecithins in water show a pretransitional exotherm on DSC thermograms which has been variously ascribed to changes in the head group or a head group/water organization or to a chain rotation or tilting phenomena. Fig. 2 shows the heating thermograms for DPPC and DHPC, which in the series of lipids under investigation here are the only ones to show a pretransition.

It has been observed previously that lower concentrations of DMPE
Figure 2

Tracings of the DSC heating thermograms of aqueous dispersions of DPPC and DHPC.
in DPPC and DPPE in DPPC cause the abolition of the pretransition (33, 59). We wished to systematically examine the effect of head group changes over a narrow range to determine what minimal variation from the PC head group is required to affect the pretransition.

Fig. 3 shows the typical effect of an admixed lipid, in this case lyso PC, on the pretransition of DPPC. It should be noted that the effects of lyso PC on the phase behaviour of DPPC has been investigated previously using differential scanning calorimetry (70).

In the presence of 5 mole % lyso PC the pretransitional endotherm is substantially broadened indicating a considerable reduction in the cooperativity of the associated phase change. At 10 mole % lyso PC, the pretransition is no longer detectable. The enthalpy of the pretransition relative to the main transition is reduced by varying percentages compared to that obtained with DPPC alone, depending on which lipid is added. Table 2 summarizes the effects of various added lipids on the pretransition of DPPC. Dipalmitin was chosen for study because it is a diglyceride, containing the same fatty acyl chains as DPPC. At concentrations between 2 - 5 mole % this compound diminishes the cooperativity and enthalpy of the pretransition and at 10 mole % the pretransition is abolished. When mixed in the lecithin bilayer at 10 mole %, DPPA abolishes the pretransitional endotherm. This phospholipid lacks the choline moiety but is otherwise identical to DPPC. Lyso PC contains no palmitic acid residue at the sn-2 carbon of the glycerol backbone and its effect on the pretransition in DPPC bilayers has been stated previously. PMPC is an interesting compound in that it is virtually identical to DPPC. Instead of a palmitic acid
Figure 3.

Tracings of the DSC heating thermograms of DPPC-water dispersions showing the effect of added lyso PC. The numbers refer to molar ratios.
### Table 2

Lipids Affecting the Pretransitions of DPPC-Water Dispersions

<table>
<thead>
<tr>
<th>Lipid Added</th>
<th>Concentration in the bilayer (mole %)</th>
<th>Effect on the pretransition&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipalmitin</td>
<td>.2</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>DPPA</td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>Lyso PC</td>
<td>2</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>PMPC</td>
<td>6</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>A&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>MM-DPPE</td>
<td>5</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>DM-DPPE</td>
<td>10</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>A</td>
</tr>
<tr>
<td>DHPC</td>
<td>10</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>A</td>
</tr>
</tbody>
</table>

<sup>1</sup> P - the pretransition is present and appears normal  
D - the cooperativity and enthalpy of the pretransition are diminished  
A - the pretransition is abolished

<sup>2</sup> A substantially diminished pretransition could be observed on initial heating but which disappeared on repeating the heating and cooling cycle.
(C₁₆) residue there is a myristic acid (C₁₄) at the sn-2 glycerol carbon. Despite this very minor structural change it has been observed that pure PMPC water dispersions show no detectable pretransitions, even though it is a disaturated lecithin. Since DPPC also shows a pretransition (30) the common point would appear to be saturated chains of equal length. When included in DPPC bilayers at 6 mole % it diminishes the observed pretransition. At 10 and 13 mole % a substantially diminished pretransition could be observed on initial heating but which disappeared on subsequent cooling and reheating. MM-DPPE has only one methyl (CH₃-) group on the ethanolamine portion of the head group as opposed to the three methyl groups in DPPC. As observed with the previous compounds, a concentration of 5 mole % diminishes the pretransition while 10 mole % abolishes it. DM-DPPE and DHPC show slightly different behaviour. DM-DPPE has one less methyl unit on the head group than DPPC. Unlike the other compounds tested, the pretransition in DPPC bilayers is apparently unaffected by concentrations of DM-DPPE up to 10 mole %. It is, however, abolished when mixed at 20 mole %. DHPC is a compound in which only the linkage of the C₁₆ chain residues to the glycerol backbone is different than that in DPPC. This lipid at concentrations up to 10 mole % caused a diminishing of the DPPC pretransition which was only abolished at higher concentrations (50 mole %).

DHPC-water dispersions also display a minor or pretransitional endotherm on heating which occurs slightly further below the main transition (8.8°) than the one for DPPC (7.5°). The effects on the DHPC pretransition after addition of a number of structurally similar
lipids are presented in Table 3. DHPE, MM-DHPE, and DM-DHPE were chosen for investigation because apart from very minor differences in the head group region they are almost identical to DHPC. Inclusion of either of these three lipids at concentrations of 8 mole % yielded substantially diminished pretransitions, while at 10 mole % any one of these compounds abolished the DHPC pretransition. The presence of 10 mole % DPPC in DHPC bilayers had no apparent effect on the pretransition however at higher concentrations (50 mole %) the pretransition was no longer detectable.

In addition to mixing the lipids mentioned in Tables 2 and 3 with DPPC or DHPC respectively, a number of water soluble additives were tested for their effects on the pretransition in DPPC and DHPC bilayers. Whereas the lipid components mixed with the lecithins might be expected to affect the chains and/or head group, the water soluble compounds would be expected to interact predominantly at the polar head group.

Acetylcholine chloride, choline chloride and ethanolamine included in DPPC dispersions so as to be at 5, 10 and 20 mole % with respect to DPPC did not significantly alter the pretransition of DPPC. Acetylcholine chloride has also been mixed with DPPC in organic solution in the same way as the mixed lipid systems were obtained, with no appreciable effects on the pretransition up to 15 mole % acetylcholine. Cooling thermograms obtained in 20 mole % of these water-soluble compounds did display a new phenomenon. As is shown in Fig. 4, cooling thermograms of DPPC-water dispersions show only one exotherm, presumably attributable to the ordering of the chains. In
<table>
<thead>
<tr>
<th>Lipid Added</th>
<th>Concentration in the bilayer (mole %)</th>
<th>Pretransition of DHPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPE</td>
<td>8</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>DM-DHPE</td>
<td>8</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>DM-DHPE</td>
<td>8</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
</tr>
<tr>
<td>DPPC</td>
<td>10</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 4.

Tracings of the DSC cooling thermograms of DPPC-water dispersions showing the effect of the presence of ethanolamine. The numbers refer to molar ratios.
DPPC : ETHANOLAMINE
(80 : 20)
the presence of 20 mole % of acetylcholine chloride, choline chloride, or ethanolamine a new small exothermal transition occurs some 12° K below the main exotherm. When samples exhibiting this minor exotherm were cooled to a point below the normal endothermic pretransition but above the minor exotherm, then reheated, no pretransitional endotherm was observed. Similar effects on the pretransitional endotherm and on the appearance of the minor exotherm on cooling have been observed with DPPC dispersed in NaCl where NaCl is at 20 mole % with respect to DPPC. This minor exotherm is broader (less cooperative) than the pretransitional endotherm.

THE EFFECTS OF DESIPRAMINE ON SINGLE LIPID DISPERSIONS

It has been shown previously that the particular phase of the lipids plays an important role in the activities of many membrane processes such as enzyme behaviour and transport phenomenon (3, 4, 5). In biological membranes several compounds have been shown to affect lipid phase transitions, e.g., cholesterol (31), proteins (30), and ions (32). Externally added compounds have also been shown to modify the phase characteristics of lipids. Among the latter are included anesthetics and tranquilizers (57) and drugs such as morphine and the tricyclic antidepressants imipramine and desipramine (34). A systematic study of the effects of desipramine on similar lipids was undertaken to determine the effect of this drug on the phase behaviour of these lipids.

It should be mentioned that desipramine alone and aqueous solutions of this drug show no observable transitions over the temperature ranges employed in these experiments.
Fig. 5 shows the typical effects of desipramine on pure lipid systems. Heating thermograms of DPPC and DPPE are shown along with the thermograms of equimolar lipid:desipramine mixes. The transition temperatures of both lipids are lowered in the presence of desipramine with the extent of lowering of the Tc being greater with DPPE than DPPC ($\gamma_{23}^0 K$ vs. $\gamma_{11}^0 K$). These results are representative of the behaviour observed with both the diester and diether series of pure lipids. The effects of desipramine on the phase properties of the pure lipids studied in section I are summarized in Tables 4 and 5.

Equimolar lipid-drug (1:1) ratios were employed since these mixes exhibit the maximum effect of desipramine on the phase properties of the lipids. With the exception of DHPC inclusion of the drug in the bilayer increases the enthalpy of transition. Desipramine also causes a reduction in the gel to liquid crystalline phase transition temperature (Tc) in all cases. The extent to which the Tc is lowered is dependent on the phospholipid head group. Phosphatidylethanolamines and their monomethyl derivatives are apparently more susceptible to the effect of desipramine than the other lipids in these series. Tc shifts of 21.7 to 24.3 K are observed with these compounds (Table 5). As methylation of the PE head group is increased, the degree of lowering of the transition temperature is decreased. Therefore the effect of desipramine on the Tc of these lipids is minimized with the phosphatidylcholine entities in both the ether and ester series. Temperature shifts of about one-third and one-half the values of their PE or MM-PE derivatives are observed for DHPC and DPPC respectively. As previously mentioned the transitional enthalpy is substantially increased by the
Figure 5.

Tracings of the DSC heating thermograms of aqueous dispersions of DPPC and DPPE showing the effect of desipramine. The numbers refer to molar ratios.
## TABLE 4

EFFECT OF DESIPRAMINE ON TRANSITION TEMPERATURE (Tc) AND ENTHALPY OF SINGLE LIPID SYSTEMS

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Transition Temperature (°K)</th>
<th>Enthalpy (kcal/mole phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Desipramine</td>
<td>+Desipramine</td>
</tr>
<tr>
<td>aDHPE</td>
<td>341.3 ± 1.5†</td>
<td>317.0 ± 1.4†</td>
</tr>
<tr>
<td>MM-DHPE</td>
<td>335.2 ± 1.3</td>
<td>311.0 ± 0.2</td>
</tr>
<tr>
<td>DM-DHPE</td>
<td>324.0 ± 1.2</td>
<td>310.0 ± 0.8</td>
</tr>
<tr>
<td>DHPC</td>
<td>316.6 ± 1.2</td>
<td>308.7 ± 0.4</td>
</tr>
<tr>
<td>bDPPE</td>
<td>336.7 ± 1.2</td>
<td>313.9 ± 0.9</td>
</tr>
<tr>
<td>MM-DPPE</td>
<td>331.4 ± 1.2</td>
<td>309.7 ± 1.2</td>
</tr>
<tr>
<td>DM-DPPE</td>
<td>321.2 ± 0.7</td>
<td>304.1 ± 0.7</td>
</tr>
<tr>
<td>DPPC</td>
<td>313.5 ± 0.5</td>
<td>302.3 ± 0.8</td>
</tr>
</tbody>
</table>

† Values reported as mean values ± 95% confidence limits (N > 4).
* All lipid:desipramine ratios are 1:1 on a molar basis.
a Diether lipid series.
b Diester lipid series.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Transition temperature changes* (°K)</th>
<th>Enthalpy changes* (kcal/mole phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aDHPE</td>
<td>24.3</td>
<td>-3.15</td>
</tr>
<tr>
<td>MM-DHPE</td>
<td>24.2</td>
<td>-1.47</td>
</tr>
<tr>
<td>DM-DHPE</td>
<td>14.0</td>
<td>-1.39</td>
</tr>
<tr>
<td>DHPG</td>
<td>7.9</td>
<td>+0.56</td>
</tr>
<tr>
<td>bDPPE</td>
<td>22.8</td>
<td>-4.14</td>
</tr>
<tr>
<td>MM-DPPE</td>
<td>21.7</td>
<td>-4.29</td>
</tr>
<tr>
<td>DM-DPPE</td>
<td>17.1</td>
<td>-0.90</td>
</tr>
<tr>
<td>DPPG</td>
<td>11.2</td>
<td>-3.24</td>
</tr>
</tbody>
</table>

* This refers to the Tc/enthalpy of the single lipid minus the Tc/enthalpy of the lipid-drug complex (see also Table 4).

a Diether lipid series.
b Diestar lipid series.
presence of desipramine in the bilayer. Our value of 11.74 kcal/mole for the DPPC:desipramine (1:1) mix differs from that reported by Cater et al. of 9.9 kcal/mole. The enthalpy shifts (Table 5) show no systematic pattern and they tend to be greater with the ester lipids than with analogous ether lipids.

PHASE SEPARATIONS INDUCED BY DESIPRAMINE IN BINARY LIPID MIXTURES

The first work on phase separation of lipid water systems was done on binary mixtures of lecithins using calorimetry (23). Since then there have been many studies dealing with phase separations in model and biological membranes. In particular, phase diagrams representing lateral phase separations in the plane of model lipid bilayer membranes have been obtained (35, 36), and transbilayer asymmetry has been observed in sonicated phospholipid vesicles (38). The heterogeneity of biological membranes has also been observed using electron spin resonance experiments (17).

As already stated in the Results section, many compounds can interact with lipids to change their phase behaviour. In essence, these molecules may represent a trigger mechanism whereby local change of fluidity or phase separation and therefore changes in the membrane permeability characteristics may also take place as a result of such interactions. Trigger mechanisms of this type could be particularly important, leading to lateral information transfer along the cell membrane.

In this respect a study of the phase characteristics of mixed lipid systems would be appropriate to the understanding of events in a
biological membrane. In particular it would be of interest to study the interaction of desipramine with mixed lipid systems and to note any differential effects upon the lipids.

When analyzed calorimetrically, binary mixtures of lipids usually exhibit a broadened somewhat uncooperative phase change relative to a pure lipid transition. Fig. 6 shows the typical melting behaviour of an equimolar mix of DPPC and DPPE. The observed broadening is due to a complete mixing of the two lipids in the gel phase resulting in an uncooperative melting phenomenon rather than two sharply distinct phase changes. If desipramine is included in the DPPC:DPPE mix such that the total lipid:drug ratio is 2:1 then the phase behaviour of the system is altered and this can also be seen in Fig. 6. Desipramine lowers the transition temperature of the mixture by approximately 12 to 13°C K and would appear to induce some sort of phase separation. At the lower end of the transition there is a fairly cooperative component which extends into a broader, less cooperative second transition. The cooling curve of this system also exhibits two distinct transitions.

Monotectics of varying lipid compositions were analyzed by DSC and these results are presented in Table 6. Again, it is observed that the transition temperatures of the PE's are affected more so than those of the PC's or DM-DMPE derivatives of a given monotectic. Sections I and II of this table refer to monotectics of different PE's and PE's and in each case the transition temperature of the mix is substantially lowered by the inclusion of desipramine and two distinct phase changes are observed on the thermograms. Unlike pure lipid systems where desipramine raises the enthalpies in most cases the enthalpies of the
Figure 6.

Tracings of the DSC thermograms of an equimolar DPPC:DPPE mixture showing the effects of added desipramine. Tracings of the DSC heating thermograms of a DPPC:DPPE (1:1) mix, curve (a), and DPPC:DPPE:Desipramine (1:1:1) mix, curve (b), showing the effects of desipramine on the pure lipid mixture.

A DSC tracing of the cooling thermogram of the DPPC:DPPE; Desipramine (1:1:1) mix is also shown, curve (c).

The numbers refer to molar ratios.
TABLE 6

EFFECTS OF DESIPRAMINE ON THE TRANSITION TEMPERATURE AND ENTHALPY DATA OF BINARY MIXTURES OF LIPIDS WHICH FORM SOLID SOLUTIONS

<table>
<thead>
<tr>
<th>Lipid system</th>
<th>Transition temperature (°K)</th>
<th>Enthalpy (kcal/mole phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPC</td>
<td>326.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>DSPC:Desipramine†</td>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td>DLPE</td>
<td>303.2</td>
<td>N.D.</td>
</tr>
<tr>
<td>DLPE:Desipramine†</td>
<td></td>
<td>4.45</td>
</tr>
<tr>
<td>DSPC:DLPE*</td>
<td>305.6</td>
<td>10.08</td>
</tr>
<tr>
<td>DSPC:DLPE:Desipramine#</td>
<td></td>
<td>15.54</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>313.5</td>
<td>8.50</td>
</tr>
<tr>
<td>DPPC:Desipramine†</td>
<td></td>
<td>11.74</td>
</tr>
<tr>
<td>DPPE</td>
<td>336.7</td>
<td>8.79</td>
</tr>
<tr>
<td>DPPE:Desipramine†</td>
<td></td>
<td>12.93</td>
</tr>
<tr>
<td>DPPC:DPPE*</td>
<td>317.0</td>
<td>10.15</td>
</tr>
<tr>
<td>DPPC:DPPE:Desipramine#</td>
<td></td>
<td>9.63</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPE</td>
<td>322.1</td>
<td>7.03</td>
</tr>
<tr>
<td>DMPE:Desipramine†</td>
<td></td>
<td>7.10</td>
</tr>
<tr>
<td>DM-DPPE</td>
<td>321.6</td>
<td>10.00</td>
</tr>
<tr>
<td>DM-DPPE:Desipramine†</td>
<td></td>
<td>9.93</td>
</tr>
<tr>
<td>DMPE:DM-DPPE*</td>
<td>322.4</td>
<td>7.64</td>
</tr>
<tr>
<td>DMPE:DM-DPPE:Desipramine#</td>
<td></td>
<td>6.44</td>
</tr>
</tbody>
</table>

† Lipid:desipramine ratios are 1:1 on a molar basis.
* Lipid mixtures are 1:1 on a molar basis.
# Total lipid:desipramine ratios are 2:1 on a molar basis.
N.D. - Not Determined.
† See Appendix I.
monotectic mixes are not systematically affected by the presence of the drug. Section III is discussed later.

THE PREFERENTIAL INTERACTION OF DESIPRAMINE WITH THE MORE FLUID COMPONENT OF MONOTECTIC LIPID MIXTURES

When the transition temperatures of two pure mixed lipids vary by \(-20^\circ\) K, there is usually no mixing of these lipids in the gel phase and two separate phase transitions are observed calorimetrically. The effects of desipramine on several of these monotectics with no solid solutions has been investigated and some typical thermograms are presented in Fig. 7. Although the upper DSPE phase change is somewhat broadened, the mixture of DLPE:DSPE (1:1) shows two distinct transitions. Immediately below this tracing is shown the same system in which the total lipid:desipramine ratio is 2:1. It would appear that the drug has a preference for the lower melting component of the monotectic as its transition temperature is substantially lowered while the upper melting component, in this case DSPE, is largely unaffected. This was found to be the case with all such monotectics investigated, Fig. 7, and this data is summarized in Table 7. In general it would seem that desipramine exhibits a preference for the lower melting component in each mix. If two PC's are mixed the transition temperature of both is lowered with the more fluid lipid being shifted to a greater extent. If a PC and a PE or two PE's are mixed, the drug will almost exclusively interact with the lower melting component. An additional lipid mix was analyzed to ensure that the preference of the drug was for the more fluid component of a monotectic and not for a particular head group. DMPE and DM-DPPE have transition temperatures less than one degree Kelvin
Figure 7.

Tracings of the DSC heating thermograms of monotectic lipid mixtures which do not form solid solutions showing the effect of desipramine on these mixtures. Numbers refer to molar ratios. The large endotherm in some traces is the ice to water phase change (at ~270° K). In the remaining traces, the water endotherm would normally mask the much smaller lower lipid phase transition (the upper lipid transition would still be observable, however). Therefore, in order to visualize the lower lipid transition these systems were supercooled to ~260° K and then reheated to yield the observed thermograms. Such a technique is useful since the water doesn't supercool (i.e., water + ice) until a temperature of ~255° K is reached. Thus, in the above systems, the water is still fluid at 260° K and when reheated from that temperature there is no water endotherm to mask the lipid or lipid-drug phase transition.
### Table 7

**Effects of Desipramine on the Transition Temperature and Enthalpy Data of Binary Mixtures of Lipids Which Do Not Form Solid Solutions**

<table>
<thead>
<tr>
<th>Lipid System</th>
<th>Transition temperature $^\circ K$</th>
<th>Transition temperature changes $^\circ K$</th>
<th>Enthalpy (kcal/mole phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC:DSPC*</td>
<td>271.1 / 306.6</td>
<td></td>
<td>1.14 / 6.43</td>
</tr>
<tr>
<td>DLPC:DSPC:Desipramine$^g$</td>
<td>252.3 / 299.0</td>
<td>18.8 / 7.6</td>
<td>1.72 / 6.18</td>
</tr>
<tr>
<td>DLPE:DSPE*</td>
<td>302.7 / 316.1</td>
<td></td>
<td>2.36 / 5.61</td>
</tr>
<tr>
<td>DLPE:DSPE:Desipramine$^g$</td>
<td>277.5 / 316.1</td>
<td>25.2 / 0.0</td>
<td>3.03 / 6.95</td>
</tr>
<tr>
<td>DLPC:DSPE$^g$</td>
<td>270 / 322.3</td>
<td></td>
<td>2.64 / 5.28</td>
</tr>
<tr>
<td>DLPC:DSPE:Desipramine$^g$</td>
<td>247.3 / 321.8</td>
<td>22.7 / 0.5</td>
<td>2.39 / 4.93</td>
</tr>
</tbody>
</table>

$^+$ Transition temperature of each individual lipid phase change.

$^g$ This refers to the value of the transition temperature of each lipid component in the 1:1 lipid mixture minus the transition temperature of the same component measured in the lipid-drug complex.

$^+$ Enthalpy of each individual lipid transition had been measured.

* Lipid mixtures are 1:1 on a molar basis.

$^g$ Total lipid:desipramine ratios are 2:1 on a molar basis.
apart. The thermogram of a DMPE:DM-DPPE (1:1) mix is shown in Fig. 8. A fairly cooperative transition is seen for this mix. Upon interaction of the drug a lowering of the transition temperature occurs but the components are not separated, although there is some upfield tailing. This result is compatible with the previously noted preference of desipramine for the lower melting component of a mixed lipid system since both lipids, in this case, have approximately the same fluidity. The data obtained from this lipid mix is summarized in Section III, Table 5: Desipramine lowers the transition temperature of DMPE to 295.8° K and DM-DPPE to 304.9° K. The transition temperature of the mix has been lowered by 22° K to a value (300.2° K) intermediate between the temperatures of the DMPE-desipramine and DM-DPPE-desipramine complexes.
Figure 8.

Tracings of DSC heating thermograms of an equimolar DMPE:DM-DPPE mix showing the effects of added desipramine. Numbers refer to molar ratios.
DISCUSSION

STUDIES OF SINGLE LIPID BILAYERS

Studies of water dispersions of DPPC, DPPE, DHPC, DHPE and the N-Methylated intermediates of both the ester and ether series of pure lipids have shown that the main chain transition temperature varies inversely with the extent of head group methylation. The simplest explanation for this behaviour would be that increased bulk (by methyl group introduction) in the head groups of the phospholipids allows for a decreased packing density and thus lower transition temperature. It is also possible that increased methylation changes the orientation between the head groups and the plane of the bilayer with consequent changes in packing density. Monolayer studies (41, 42, 43) have indicated that PE's are more closely packed than PC's with the limiting areas per molecule for PE's being approximately 4\(\text{Å}^2\) less than for analogous PC's. Although all the lipids used would be expected to be zwitterionic over a broad pH range it has been observed that charge neutralization may occur in PE's but not in PC's because of differences in head group orientation with the bilayer in the two lipid-water systems (44). It was suggested that the PC head groups are oriented tangential to the bilayers or interdigitated in a translamellar fashion.

The transition enthalpies do not show a systematic variation in either series. Apparently, any methylation of the PE head group raises the enthalpy to a relatively constant value ranging between 9.09 to 9.53 \(\text{kcal/mole} \) phosphate. In the diester series, DM-DPPE has the largest enthalpy with the other three lipids being approximately equal.
(8.50 to 8.79 kcal/mole phosphate).

The measurements made here confirm, extend and provide enthalpy data on some of the compounds previously measured by the technique of differential thermal analysis (59). The enthalpy value for DPPC (8.50 kcal/mole phosphate) is in agreement with that found by Phillips et al. (47) and Chapman et al. (33) but lower than that reported by Hinz and Sturtevant (39) and Cater et al. (34). Applications of the type of enthalpy correction used by Hinz and Sturtevant (39) failed to significantly alter the heat of the main transition. In some cases these differences may arise where kcal/mole phosphate is measured rather than kcal/mole lipid on a weight basis. In the phosphate determination the amount of lipid present is measured after calorimetric analysis and thus may provide a more accurate value of the quantity of lipid involved in the phase transition, thus avoiding inaccuracies due to water evaporation during the sample preparation and transfer.

Perturbation of Long Range Order of Saturated Lecithins by Foreign Molecules

As stated in the Results section it has been reported previously that the disaturated lecithins in water show a pretransitional endotherm or minor transition on DSC thermograms. The pretransition has been attributed to changes in the head group or head group/water reorganization (23, 45, 46) or possibly to a chain rotation (39) or tilting phenomenon (26) or a combination of these effects. It has also been noted in the Results that the pretransition observed in model lecithin membranes is very sensitive to the presence of foreign molecules in the bilayer.
The susceptibility of the pretransitional endotherm of saturated lecithins has been observed previously with cholesterol (23), drugs (34), phosphatidylethanolamine (33, 59), and local anaesthetics (48) and lyso PC (70). With the exception of the observations in the membranes with PE and lyso PC most of the foreign molecules have been of substantially different structure. In these experiments we have attempted to determine the effects of compounds of a very similar structure on the pretransition.

The observation of the lack of a pretransition in EMPC is quite interesting. It is noted, however, that the possibility of impurities in the commercial preparation which we may not have detected could be responsible (a fatty acid analysis kindly performed by M. Hack showed a composition of 47 mole % myristate and 53 mole % palmitate with an error in determination of approximately ± 1%). Thus it may be that we have a mixed system some of which is dipalmitoyl PC. It is noted with regard to this, however, that all mixtures of DMPC and DPPC have been found to display pretransitions (33).

Perturbation of the DPPC pretransition occurs in the presence of 5 mole % of a number of lipids with the same acyl chain length (Table 2). In almost all cases the pretransition is completely removed at 10 mole % of the second lipid. Dipalmitin, DPPA, MM-DPPE and DM-DPPE do not have as large a head group as DPPC. It could be argued that increasing spacing is allowed in the head group region on the insertion of the molecules resulting in a different packing arrangement for the DPPC head group. Similarly, the effects of DHPE, MM-DHPE and DM in DHPC could result from head group packing changes. However, none
of these added compounds have a choline head group, and do not display a pretransition on their own. Chain orientation in these compounds with respect to the plane of the bilayer could be expected to be different from that of DPPC and at sufficient concentrations they may affect the ability of the DPPC chains to take up the tilted configuration in the gel state.

Lyso PC has an identical head group to DPPC, and thus the absence of the pretransition at 10 mole % would appear to be most easily accounted for by interference in chain packing. It is of interest to note with respect to the addition of lyso PC that even at 10 mole % no substantial effects on the main chain endotherm are observed (Fig. 3), in spite of the fact that lyso compounds are often considered to have disruptive effects on membranes. Klopfenstein et al. (70), have observed that up to approximately half of the bilayer can be lyso PC without substantial effects on the main-chain melting enthalpy or phase homogeneity.

DHPC alone in water displays a pretransition as does DPPC. Mixtures of these compounds do not as dramatically affect the pretransition as do the other compounds. At 1:1 molar ratios of the two lipids, however, the pretransition is no longer evident in contrast to similar mixtures of DMPC and DPPC where the pretransition is still evident although slightly broader than for the single lipids (33). The presence of ether bonds in the backbone region of DHPC may account for this difference. Similarly, very slight differences in the packing of the chains in the diether compounds may account for the observation that the pretransition is abolished in DHPC:DM-DHPE 9:1 mixtures while it is
still present in mixtures of the dipalmitoyl components at the same concentrations.

Acetylcholine, choline and ethanolamine when intimately mixed with the lipids before dispersion might be expected to pack in the head group region with possible changes in the pretransition if head group movement were its origin. However, up to 20 mole % of these compounds, no significant effect on the pretransitional endotherm is observed, indicating that these compounds are likely to remain in or are excluded to the water. It has also been found that interaction at the head group of uranyl (UO$_2^{2+}$) ions which presumably bind to the phosphate oxygens do not abolish the pretransition (33). These water soluble additives were originally intimately mixed with the lipids in organic solution to see if these compounds affected the lipid before being presumably excluded to the water phase. No observable effects were noted in the lipids and therefore in subsequent experiments the lipids were dispersed in aqueous solutions of these compounds. The induction of a minor exotherm on cooling in the presence of these compounds appears to be attributable to a reversal of the process responsible for the pretransition, since if the samples are not cooled below this minor exotherm the normal pretransition is absent on reheating. On cooling DPPC alone in water no minor transition is seen. This would indicate that the rearrangement (most likely of the chains into the tilted configuration) is either a non-cooperative process or that it occurs under the main exotherm. Although this transition can be either displaced or increased in cooperativity in the presence of 20 mole % of acetylcholine, choline and ethanolamine, it would appear that it is caused
by some long range electrostatic effect since NaCl at 20 mole % (165 mM) can also induce the same behaviour. ¹

The majority of findings reported here can be most easily and consistently accounted for if the pretransition arises at least principally from a hydrocarbon chain rearrangement, e.g., from the tilted to perpendicular configuration (26). The arrangement is sensitive to disruption by small amounts (1 molecule in 20) of added lipids of very similar in structure. (In the case of dipalmitin 1 in 50 molecules can cause disruption.) This may have interesting implications for the modulation of membrane processes, which depend strongly on lipid configuration, in that the addition of lipid soluble compounds of substantially different structure could cause long range changes at very low concentrations.

BIOLOGICAL RELEVANCE OF COMPOUNDS AFFECTING LIPID PHASE TRANSITIONS

It is now generally accepted that most biological membranes possess extended regions of phospholipids arranged in a bilayer configuration. It has also been shown that the lipid components of these membranes are capable of undergoing a change in phase from the gel state to the liquid crystal form. These phase transitions are accompanied by changes in such things as lipid mobility, diffusion properties and bound water interactions and therefore compounds that can affect or induce lipid phase transitions would be of biological importance. Many such compounds have been reported including proteins, metal ions, steroids, lipids, anaesthetics and drugs. Desipramine is an antidepressant drug that has been the subject of this work.
Many events that occur in biological membranes, for example, transport processes or enzyme activity, have been shown to be related to the physical state of the membrane lipids. Therefore, the compounds mentioned previously, including desipramine, could play a significant biological role if they were to interact with such membranes, affecting the lipid components in such a way as to change the local membrane fluidity or induce varying degrees of phase separation. Desipramine has been found to lower phase transition temperatures of a number of lipid types with the extent of depression being dependent on the lipid head group. It has also been observed that in mixed lipid systems the drug appears to preferentially interact with one component, the lower melting component. Similar preferential interaction has been observed for cholesterol in mixed lipid systems (31). In contrast to cholesterol, however, desipramine appears to influence the phase behaviour without substantial reduction in enthalpy and without the drastic loss in cooperativity observed in the presence of cholesterol.

SOME POSSIBLE MECHANISMS FOR THE ACTION OF DESIPRAMINE IN LIPID BILAYERS

With respect to pure lipid bilayers at least three possible mechanisms for the action of desipramine may be considered.

Firstly, desipramine may interact at the phospholipid head group—the nitrogen of the amino propyl group hydrogen bonding with the phosphate oxygens. As noted previously the ability of desipramine to lower the transition temperature is somewhat dependent on the nature of the head group. Therefore a larger head group, e.g., choline, may cause
some shielding of the phosphate oxygens and the effects of desipramine are lessened relative to an ethanolamine head group. Not only size but head group orientation may also play a role in the ability of desipramine to interact with the lipid. It has been reported previously (44) that PC head groups are oriented perpendicular to the plane of the bilayer whereas PE headgroups are arranged parallel or tangential to the bilayer. It is possible that the two N-methylated derivatives take up intermediate orientations with respect to the bilayer and that the effects of the drug are dependent on the particular orientation involved. In an artificial PE bilayer the zwitterionic head groups are kept in close array by a dipolar interaction between the ethanolamine amino group and the adjacent phosphate oxygen(s). It is also known (44) that PE head groups undergo motions which are only one-half as mobile as those for corresponding PC's. Therefore if desipramine hydrogen bonds to the oxygens of the phosphate group, the dipolar interactions between adjacent PE's may be weakened or broken resulting in a more mobile head group region with a consequent drop in transition temperature. Since PC's are more mobile the effects of desipramine in lecithin bilayers would not be as marked. With respect to the enthalpy changes it is possible that a drug-lipid complex could involve a structural reorganization such that a greater (or in the case of DHPC, a lesser) amount of energy is required to induce an order → disorder transition in the complex. This being the case it would seem that a desipramine-ester lipid would form a very stable complex relative to the pure lipid with the ether lipids forming a somewhat less stable complex.

Alternatively desipramine may hydrogen bond at the head group or
glycerol backbone region (i.e., ester or ether oxygens) with partial penetration into the bilayer. There are, however, arguments against this type of interaction. The fluidity or mobility of the hydrocarbon chains is greatest at the methyl terminus and decreases as you approach the backbone region (71). If desipramine were partially solubilized in the hydrocarbon region it would tend to fluidize the chains in the gel state with a concomitant decrease in the enthalpy as was shown to be the case with cholesterol (23). Generally speaking this was not observed (DHPC excepted). The absence of a potential hydrogen bonding species (ester carbonyl oxygen) in the ether series is apparently of little significance when comparing the Tc shifts of desipramine in both ester and ether lipids. Preliminary results from temperature jump experiments have indicated little or no difference in transition kinetics between pure lipid and lipid-drug systems (72).

As a third possibility that might be considered is that the drug exerts its effect on the lipids by in some way altering the water structure in the immediate region of the bilayers. Desipramine is soluble in water and organic solvents and its exclusion to water in the systems under study may cause some structural short range reorganization to occur in the water phase. This in turn may cause a reorganization of the lipid order. Structural changes associated with a hydrophobic effect have been discussed previously (73). It is suggested that "water molecules are ordered into networks forming cagelike cavities within which non-polar solutes may be enclosed". On the addition of water to phospholipids, the temperature at which the phase transition occurs is lowered and it reaches a limiting value when the maximum concentration
of bound water (20% for PC) is achieved (23).

In an attempt to shed further light on this question preliminary experiments have been attempted with the drug-lipid suspensions to see if any changes in the enthalpy of the ice-water transition can be affected by the presence of the drug. Differences have been noted between the enthalpy of the water melt in the lipid-water dispersions in comparison with the dispersions containing drug, but consistent reproducible behaviour has not been established. The high sensitivity of the DSC-2 and the large heat capacity of water require that extremely small samples be used, and the problem of evaporation during handling is severe. Alternate ways of examining this possibility using partitioning of car spin labels or through pulsed nmr are being considered.

THE ACTION OF DESIPRAMINE ON MIXED LIPID SYSTEMS

The interaction of desipramine with mixed lipid systems would be consistent with a preferential interaction of the drug with one of the components. The action would appear to be such that the lower melting of the two components is preferentially shifted. The shifts of the lower melting components are consistent with those found for pure lipids. For example, desipramine lowers the \( T_c \) for pure DLPE by 29° K and in a 1:1 DLPE/DSPE mixture the drug shifts the transition temperature of DLPE by 25° K.

The effects on the enthalpies of the individual induced or shifted transitions in the mixed lipid systems are small, consistent with the observation of the effects of the drug on pure components of the same binary lipid mixture. This behaviour is different than that observed with
pure single lipid systems where desipramine significantly raises the enthalpy in most cases (1.39 to 4.29 kcal/mole phosphate), Table 4. DM-DPPE shows a slightly lower increase (0.90 kcal/mole phosphate) while DHPC shows a decrease in enthalpy. It should be noted here that our enthalpy value for pure DPPC is lower than that reported by Cater et al. (8.5 kcal vs 9.4 kcal) but the enthalpy of DPPC:desipramine (1:1) is higher (11.7 kcal vs 9.9 kcal).

Exact quantitation of enthalpies in mixed systems is more difficult than with single, pure components because of the difficulties encountered in determining the exact points of departure of the tracings from the baseline. With respect to the monotectics with solid solutions we have seen the ability of desipramine to induce phase separation (Fig. 6). This type of behaviour would tend to indicate a lack of or only a small amount of solid solutions in the two components. This would indicate the presence of two separated components in the gel phase and could be explained by a drug-lipid component and a simple lipid component. Cooling thermograms of this mix are also bimodal indicating that separation of the two components remains in the liquid crystal phase. It is also possible that the formation of separate vesicles (a drug-lipid and a simple lipid) may account for the observed results.

POSSIBLE PHARMACOLOGICAL EFFECTS OF DESIPRAMINE

As mentioned earlier (3, 4, 5), the activities of many membrane processes has been shown to be related to the physical state of the lipids in the membrane. In biological membranes many compounds have been known to affect lipid phase transitions (30, 31, 32, 34, 57) and included among these is the antidepressant drug, desipramine. The exact
mode of action of this drug is as yet unknown but it has been shown to alter the phase characteristics of many lipids. The selective interaction of cholesterol with the more fluid lipid in mixed lipid systems has been observed and the effects on the lipid phase behaviour noted (31). Cholesterol causes a drastic reduction in the enthalpy of the phase transition without markedly affecting the transition temperature. Desipramine, however, when interacted with single or mixed lipid systems causes a lowering of the transition temperature without substantial alteration of the enthalpies. It would therefore appear that desipramine and cholesterol act via different mechanisms. Since cholesterol would be expected to solubilize mainly in the hydrocarbon chain, desipramine would appear to be exerting its effects through some sort of lipid head group interaction.

It is possible that some of the pharmacological effects of desipramine may derive from its ability to alter membrane fluidity or to induce phase separation or both. Desipramine is known not to act as a monoamine oxidase inhibitor but to block the transport of noradrenalin apparently by inhibiting the amine transport mechanism in the neuronal membrane (51, 52). On the basis of the x-ray structure of imipramine (53), which is the dimethyl amino propyl analog of desipramine, it has been suggested that such inhibition may occur by the binding of the dimethyl amino propyl side chain of imipramine to the same sites as those occupied by the putative CNS neurotransmitters noradrenalin (NA) and 5-hydroxytryptamine (5HT). Desipramine which has a very similar side chain could be anticipated to act in a similar
fashion. In addition to the terminal amino group of the side chain blocking the binding site with which the primary amine portion of NA or 5HT normally interacts, the possibility of one of the benzene rings of the tricyclic nucleus blocking the binding site usually occupied by the aromatic ring of NA cannot be ruled out (54). Similarly a larger part of the tricyclic system may effectively block the binding site of the indole nucleus of 5HT.

In the absence of any specific binding information and on the evidence noted above for dependence on the physical state of lipids of a number of membrane-associated processes, it may be reasonable to suggest that the effects of desipramine could be due to similar effects on lipid fluidity or on phase separation. These could be exerted either by changing the activation energy for transport or by disrupting the normal distribution or orientation of receptor sites in the membrane for NA. It is noted in this respect that a number of local and inhalation anaesthetics have been found to alter fluidity in biological and artificial membranes (48, 53, 56, 57, 74, 75).

Although large amounts of drug have been used in these studies to maximize the observed effects it is noted that desipramine concentrations as low as 2 mole % can alter the phase transitions of DPPC (54). While most drugs appear to be metabolized by detoxifying enzymes in the liver with subsequent excretion of substantial amounts of the metabolites, it is of considerable interest that in the case of antidepressants prolonged administration is necessary to achieve the desired therapeutic effect (58). It has also been observed that the
administration of antidepressants over a period of time (4 - 8 weeks) is employed when measuring the effects of these drugs on the response to NA of the cyclic AMP generating system in brain tissue (49). Because of the possibility of selective accumulation of any drug in specific tissues the exact concentration at the site of action is open to question. However, it may be in the case of desipramine that enough time is required for sufficient accumulation of the drug in the neuronal membrane to permanently alter the lipid structure with consequent physiological changes.
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APPENDIX I

For sharply defined transitions involving standards or highly co-operative lipid samples the transition temperature is determined by the point of intersection of a tangent to the leading edge of the transition and the extrapolated baseline. See arrow in tracing (a).

In the same transition in the presence of desipramine the endotherms often showed an initial uncooperative shoulder prior to a fairly cooperative main melt. The determination of the exact point of baseline departure for uncooperative endotherms is difficult (23), and so to obtain a reproducible and conservative estimate of the drug-induced shift, Tc's were measured on the main endotherm. See arrow in tracing (b).
(A) Structural formula representative of the diester lipids used in this study.

(B) Structural formula representative of the diether lipids used in this study.

\[
\begin{align*}
\text{PE} & \quad R_1 = R_2 = R_3 = H \\
\text{N-methyl PE} & \quad R_1 = \text{CH}_3, \quad R_2 = R_3 = H \\
\text{N,N-dimethyl PE} & \quad R_1 = R_2 = \text{CH}_3, \quad R_3 = H \\
\text{PC} & \quad R_1 = R_2 = R_3 = \text{CH}_3
\end{align*}
\]

(C) Structural formula of Desipramine Hydrochloride.
APPENDIX II

(A) \[ \text{H}_2\text{C} = \text{O} = \text{C} - (\text{CH}_2)_n - \text{CH}_3 \]

(B) \[ \text{H}_2\text{C} = \text{O} - \text{CH}_2 - (\text{CH}_2)_n - \text{CH}_3 \]

(C) [Chemical structure diagram]

\[ \text{H}_2\text{C} = \text{O} - \text{P} = \text{O} - \text{CH}_2 - \text{CH}_2 - \text{N} - \text{R} \]