VIBRATIONAL SPECTROSCOPIC STUDIES

OF

PHOSPHOLIPID PHASE TRANSITIONS

by

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A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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कर्मण्येवाधिकारस्ते मा फलेषु कदाचन |
मा कर्मफलहेतुर्भूर्मा ते सङ्गोऽस्त्वकर्मणि ||

SHRIMAD BHAGWAD GITA

To action alone hast thou a right
and never at all to its fruits;
let not the fruits of action be
thy motive; neither let there be
in thee any attachment to inaction.

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Abstract

VIBRATIONAL SPECTROSCOPIC STUDIES

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PHOSPHOLIPID PHASE TRANSITION

by

N. Krishnamachari

Adviser: Professor Bernard J. Bulkin

Infrared and Raman spectroscopy have been used extensively as tools for measuring the phase transition of model membrane systems. In both of these techniques, the number of bands observed, their relative intensities, and their band widths all yielded information of the mobility and degree of fluidity of the hydrocarbon chains of the lipid-water phases.

The ability to probe these bilayer systems without introducing any chemical perturbation, using small amounts of sample, with simultaneous examination of all parts of the phospholipids, make infrared and Raman spectroscopy important methods for the study of biomembranes.

Results are presented on neutral lipid-water gels and mixed lipid gels in the temperature range 0-80^{o}. The relationship between the charge on the polar head and the mobility of the lipid hydrocarbon tails has been explored

by examination of the influence of an anionic phospholipid, phosphotidyl serine, on the gel-liquid crystal phase transition of a lecithin-water phase. Study of the protein-lipid complex of oxidized and reduced cytochrome C with lecithin by infrared spectroscopy is also included. Some thermograms of these lipid gel transitions, light scattering and light transmission studies, are also included.

Hysteresis and cooperative aspects of the transition of these lipid gels focus attention on the long range, multimolecular interactions existing in the lipid bilayers.

INTRODUCTION

MEMBRANE STRUCTURE AND MEMBRANE MODELS

The roles played by biological membranes in the cell function are not yet well understood because of the inherent complications associated with membranes. The complications are due not only to the mere presence of giant molecules in membranes, but also to the variations in their molecular structures and organization. For example, in unicellular organisms, all vital processes occur in a single cell. But in higher animals having multicellular organisms the function of the cells are many. Cells of gastrointestinal system digest and absorb food, those of the respiratory system take up oxygen and eliminate carbon dioxide, the urinary system removes wastes, the cardiovascular system distributes food, oxygen and the products of metabolism. The above functions of those cells of multicellular organisms are not yet completely correlated with the type of membranes surrounding those cells and their multifarious functions.

As a first step, the complex nature of the cell membranes can be understood by studying their composition, molecular arrangement, dynamic character and the mutual interactions existing between molecules. Generally the biological membranes are found to contain four classes of molecules. They are: 1) lipid, 2) protein, 3) cholesterol,

and 4) water. It is noteworthy to mention that except for bacterial membranes, all other membranes contain cholesterol.

Membranes form the boundary of all cells that separate the protoplasm from its environment. By virtue of their dynamic nature they possess a wide range of biological and physiological properties. The exact definition has been discussed critically by Robertson[1] and certain physico-chemical aspects by Weiss[2] and Fernandez-Moran[3].

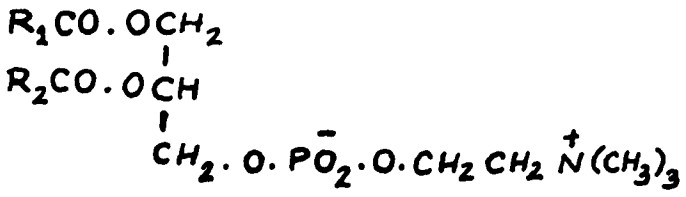
Membranes contain up to 25 to 30% by weight phospholipids. Essentially all the lipids present in membranes, excluding steroids, are found to be in the form of phospholipid. The membranes have a remarkable structure. They are not only semipermeable, allowing some substances to pass through and excluding others, but their permeability can be varied. They also possess elasticity.

The existence of the membrane was suggested, by De Vries[4] and subsequently by Pfeffer[5] and Overton[6], from experiments in plant cells. The plant cells were found to respond osmotically to variations in external osmolarity which suggested the existence of the type of semi-permeable membrane. This was only a beginning. As years progressed the advancement in chemistry made possible knowledge of the composition of cellular membranes in detail. It was believed that this would provide the information which could serve as the basis for formulation

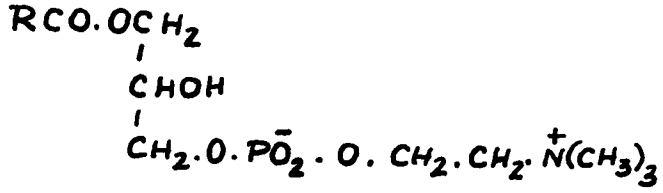
Figure 1

Structural formulae of neutral phospholipids used in this work. The R's represent hydrocarbon chains, some of which may be unsaturated.

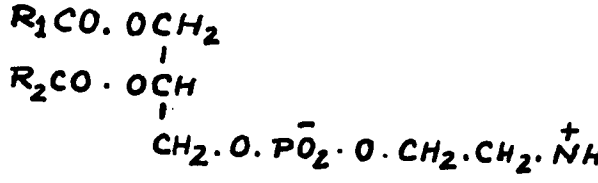
1. LECITHIN -(L) - PC



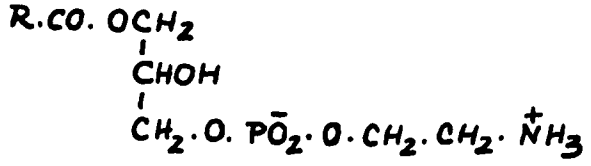
2. LYSOLECITHIN - LL



3. PHOSPHATIDYL ETHANOLAMINE - PEA



4. LYSOPHOSPHATIDYL ETHANOLAMINE - LPEA



of a model of membrane structure. However, as time passed, the need for knowledge about molecular organization of the membrane took on greater and greater importance. Many review articles [7, 8, 9] on molecular organization appeared in book form and in biological journals. All these attempted to fit the experimental facts with the proposed models. Many membrane models were suggested. Some of the more prominent of these models are discussed in the following section.

Phospholipids form the centre, life and chemical soul of bioplasm of plants as well as animals and this was recognized as early as 1884 by Thudichum[10]. It is also a recognized fact that there are no membranes without phospholipid. The basic chemical structure of phospholipid is derived from triglyceride, phosphate and choline groups. The polar character is given to the molecule by the phosphoric acid residue and the ammonium group. There are wide varieties of phospholipid present in membranes. The differences in the phospholipids arise from the number of hydrocarbon chains, their length, the degree of saturation and the net charge at the polar end of the phospholipids. The structure of one of the important and most widely distributed phospholipids in tissue, phosphatidyl choline, is given in figure 1. Phosphatidyl choline has the L-3-glycerophosphate structure. The other important phospholipids are lysophosphatidyl choline, phosphatidyl

ethanolamines (PEA), lyso PEA, and anionic phospholipid e.g., phosphatidyl serines. The detailed structures of these are also shown in figure 1.

MEMBRANE MODELS

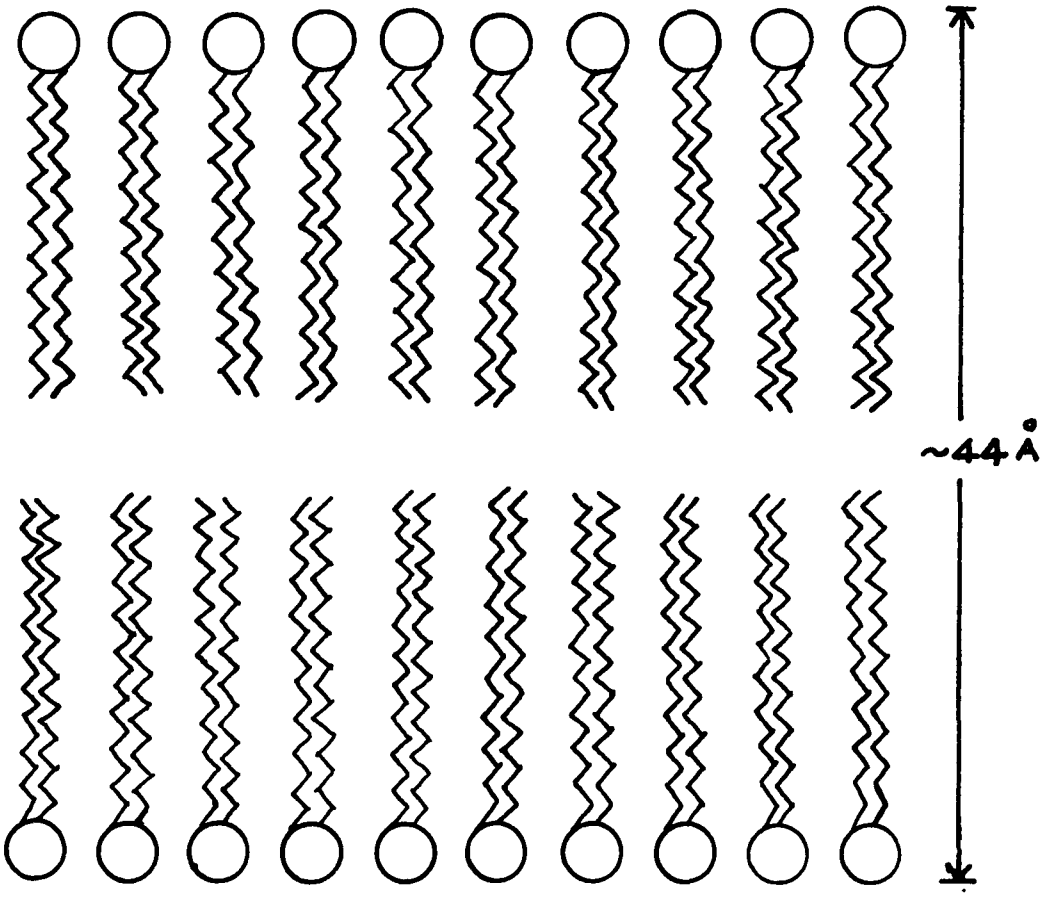
1. Lipid Bilayer Model:

The first and foremost of these membrane models suggested was the lipid bilayer model. The idea for this model was suggested by the historical experiment done by Gorter and Grendel[11] in 1925, who estimated the thickness of the erythrocyte membrane by extracting lipids from erythrocyte ghosts and spreading them over an air-water interface. Lipid molecules arrange themselves at a water-air interface with their polar moieties in the water phase and non-polar hydrocarbon tails in the air. From the area of the unimolecular lipid film and the total surface area of the erythrocytes from which it was derived, Gorter and Grendel suggested that the amount of lipid present in erythrocyte ghosts was just sufficient to provide a bimolecular leaflet. The surface area they calculated was incorrect. In addition, they did not extract the lipid completely from blood cell membranes. This experiment, however, gave both an approximate idea about the bimolecular leaflet organization of lipid in membranes and an approximate thickness of the membrane. Thus, what is now known to be their correct conclusion

Figure 2

Bilayer arrangement of lipid.

Circles represent polar heads and the tails hydrocarbon chains of lipids.


~44 Å

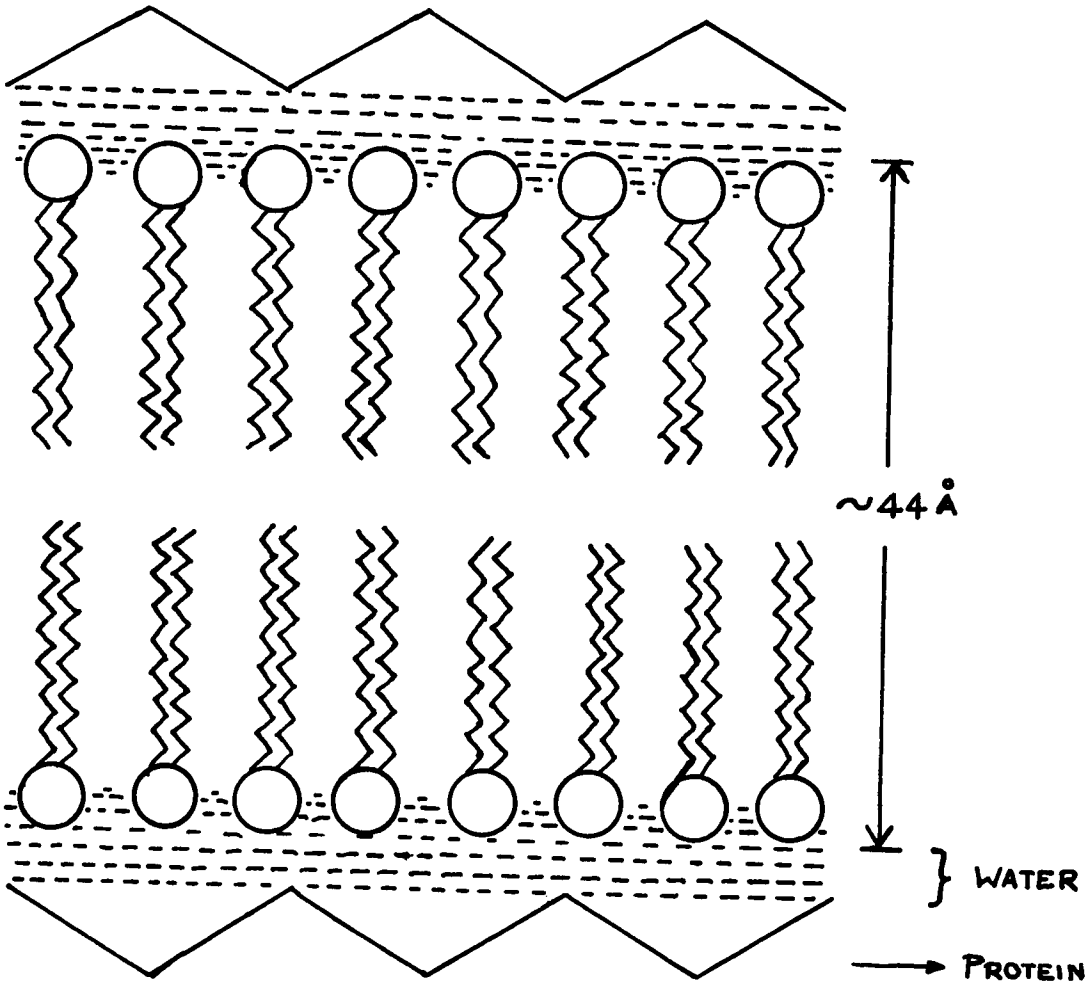
from their incorrect experiment was that the lipid is present as a double layer. Figure 2 shows the lipid bilayer. The hydrophilic portion of the lipid, consisting of phosphoryl choline, is represented by the circular dot while the hydrophobic, hydrocarbon chains of the lipid are represented by two tails. The bilayer arrangement of lipids in membranes was later proved by studies including surface chemistry, electrical measurements[12], X-ray diffraction[13] and electron microscopy[14].

This lipid bilayer arrangement was also suggested by the surface tension measurements done in 1935 by Danielli and Harvey[15]. The surface tension was found to be less than 1 dyne/cm which suggested that protein was on the surface of the membrane. This actually led Danielli and Davson[16] to propose an improved lipid bilayer model, in which the non-polar portions of the lipid molecules were directed inwards and the polar groups were directed outwards, with absorbed monolayers of hydrophilic protein as shown in figure 3. Thus, most membranes were predicted to be about 60 to 100A° thick. Danielli and Davson claim that they were unaware of Gorter and Grendel's paper in the Dutch journal.

The examination of the membranes by electron microscopy fit this model. The observations and hypotheses incorporated into this model were found to be correct and responsible for generating deep research in building membrane

Figure 3

Lipid bilayer arrangement of Danielli and Davson[16].


~44 Å
WATER
PROTEIN

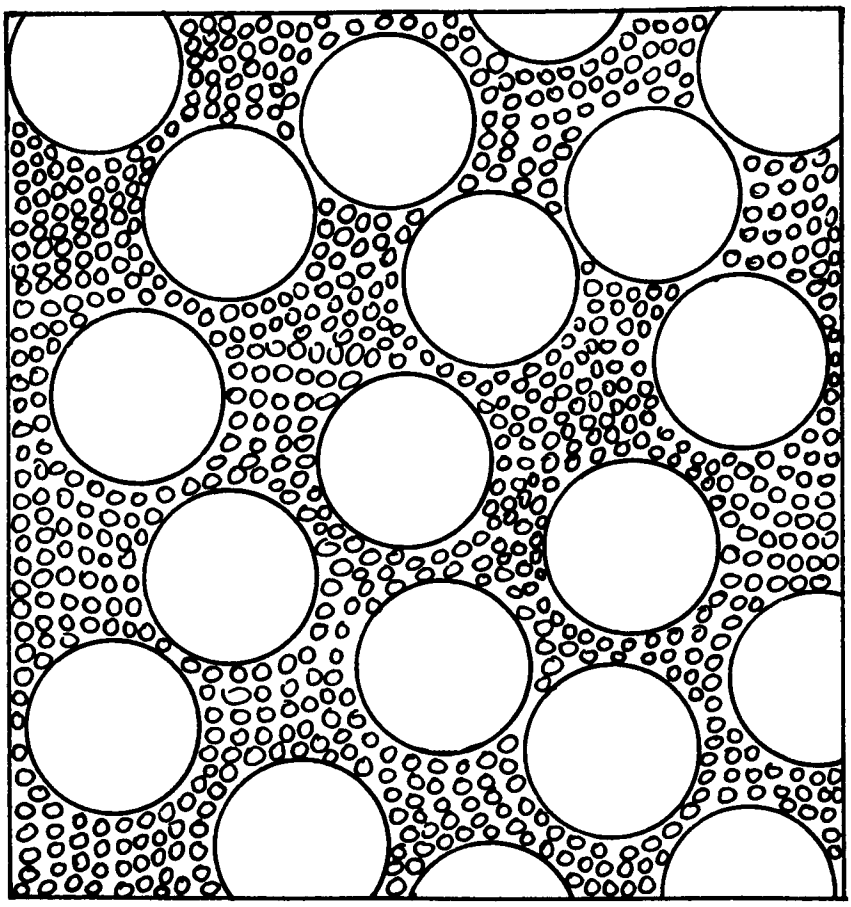
models. A 'railroad track' like arrangement was found to appear as two dark lines in the electron microscopy separated by a lighter surface[14]. Robertson considered these two dark lines as characteristic of the electron microscope image corresponding to stained protein. However, Stoeckenius[17] considered these lines as representing the hydrocarbon portion of the lipid whose unsaturated fatty acids had been stained. Later evidences[18, 19] proved that the two lines represent protein rather than hydrocarbon portion of the lipid. The total thickness of the membrane in this model thus depends on the thickness of the lipid bilayer, protein and aqueous layer.

2. Protein Liquid Crystal Model

This model was suggested by Vanderkooi and Greene[20] to include dynamic character of the membranes. This model is shown schematically in figure 4. Proteins are distributed within and outside of the lipid bilayer in such a way that the hydrophilic groups are close to the lipid polar head groups and the nonpolar amino acid side chains facing the nonpolar lipid tails. The most direct evidence for this model comes from the X-ray diffraction studies[21,22] on wet, untreated retinal disc membranes. It was found that proteins and lipids are in constant thermal motion in the plane of the membrane, rather than in a rigid crystal lattice. This model supports the view that the membrane has a dynamic rather than static character; within

Figure 4

Surface view of retinal rod disc membrane[20], showing the random arrangement of the proteins and lipids. The large circles represent the proteins and the small circles the lipids (redrawn from reference 83).



limits, lipids can be added to or removed from membranes. In the protein crystal model (figure 5) the dynamic character of the membrane has not been incorporated. This model also accounts for the surface profile obtained by X-ray diffraction[22].

The protein liquid crystal model is one of the first suggested to take into account the liquid crystalline nature of the membrane components. It is fact that phospholipids and cholesterol form liquid crystals with water. Endothermic phase transitions have been studied in much detail to prove that lipid-water-cholesterol liquid crystalline complexes exist in membranes.

Thus this protein liquid crystal model of biological membranes accounts satisfactorily for all the experimental observations on the properties of membranes and the interrelationship of lipid and protein.

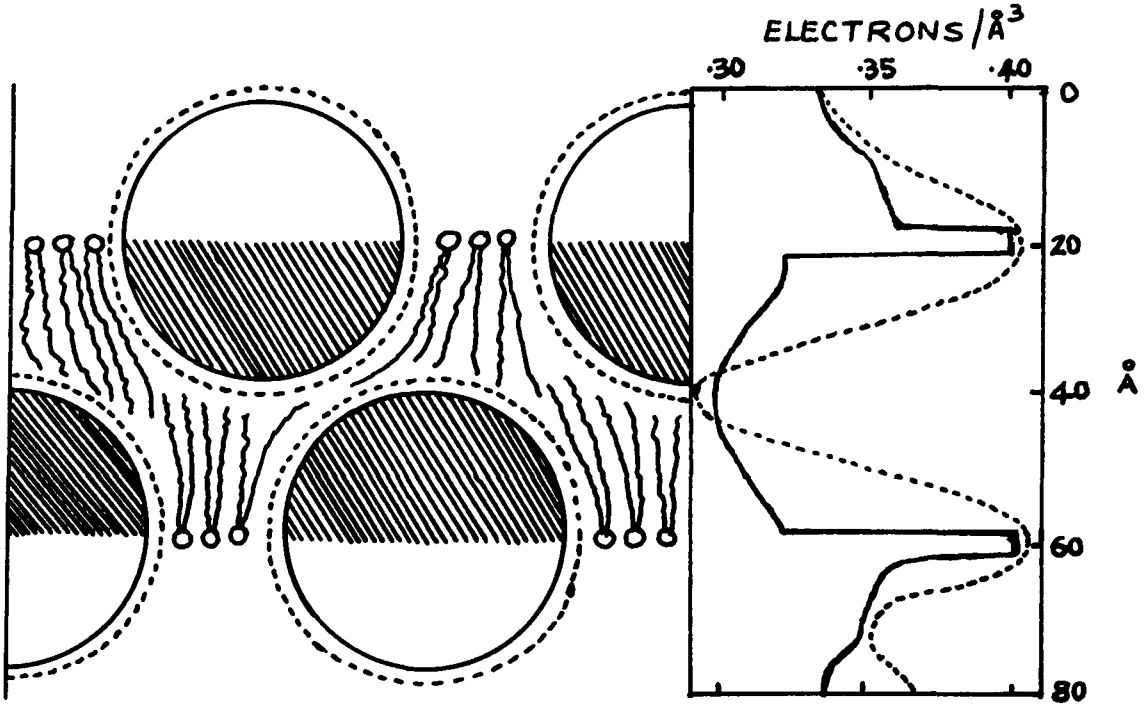
There are quite a few membrane additional models suggested in literature[23-25], each one trying to accomodate the chemical and physical properties of membranes.

The basis for the evolution of membrane structure and membrane models

Various physical techniques have been employed to elucidate the membrane structure and thereby fit those data in a form called a membrane model, such as described in the previous section. The physical techniques used

Figure 5

Section of retinal disc membrane with the calculated electron density across the membrane. The large circles represent the proteins. The small circles with tails represent phospholipids. The cross hatching in the proteins denotes a predominantly non polar surface. The solid line in the graph at right represents the calculated electron density distribution and the dashed curve is the Fourier synthesis map[29](redrawn from reference 20).


ELECTRONS/Å³
·30
·35
·40
0
20
40
60
80
Å

are numerous. Of these most important techniques are: a) X-ray diffraction b) electron microscopy c) differential thermal analysis d) nuclear magnetic resonance spectroscopy e) electron paramagnetic resonance spectroscopy and f) infrared and Raman spectroscopy.

Table I shows the time scale of various physical techniques employed to study the membrane structure. Of these infrared and Raman techniques fall in the time scale of 10^{-12} to 10^{-14} seconds, which seems to be more suitable to look at the intramolecular motions of hydrocarbon chains of lipid molecules, whereas the other techniques like electron microscopy tend to give a more averaged view of these motions.

a) X-ray diffraction: This is essentially an averaging or static technique. This technique has been used to study membranes[1, 26-28] and model systems[29-32]. The low angle X-ray diffraction furnished general information on the dimensions and approximate distribution of scattering groups. Electron densities, which are indicative of molecular organization, have been obtained[33] for myelin. X-ray diffraction of lipid-water systems has furnished information on the structure of the mesomorphic phases[34].

b) Electron microscopy: The advent of electron microscopy made it possible to elucidate the molecular organization of membranes and their derivatives, which

constitute 40-90% of the total mass in various cells. Membrane function cannot be understood without knowing the details of configuration of membranes and their relationship to each other. The high resolving power of the electron microscope made it possible to directly visualize individual cell membranes that are only 100 Å thick[35, 36]. This technique involves complex fixation and dehydration. Generally OsO_4 (Osmium tetroxide) or $KMnO_4$ (Potassium permanganate) is used for fixation of membranes. This is also an averaging technique. Electron microscopic evidence has enabled the extension and modification of the original hypothesis provided by Danielli and Davson that membranes are enveloping cells as a bimolecular leaflet of defined thickness stabilized by absorbed protein monolayer. Electron microscopes with magnification as high as half a million have been used for study of membranes. Proof for bilayer arrangement of lipids comes primarily from electron microscopy.

c) Differential thermal analysis: This technique has been used extensively to probe membranes[37, 38] and model membranes[39, 40] to study their endothermic phase transition. The transitions were found to be reversible and to occur at the same temperature on heating and cooling. By this technique one could determine the transition temperature of membranes or model membranes as well as the heat absorbed at this transition. According to

these studies the characteristic transition temperature of the lipid-water complex depends upon the length of the lipid hydrocarbon chains, the degree of unsaturation of the hydrocarbon chains, the chemical structure of the lipid polar head groups, and the water content of the system[41].

d) Nuclear magnetic resonance spectroscopy: This technique is valuable for studying various aspects of cell membranes structure and function. This is also a time averaging technique to a certain extent, thereby limiting the probe's capability to distinguish fast motions of the molecules from slow motions. (See Table I for time scale.)

Since membrane systems are complicated, consisting of lipid, protein, water, metal ions, sugar groupings and sometimes cholesterol, this technique has been predominantly used for simple model membrane systems. For such systems the interpretation becomes simpler. This technique, even though one of the most powerful tools, is not without limitation. The first limitation is the limited ability to study only part of the molecule. The large sample requirement is another. For example, we could study ^{1}H nuclei while all other nuclei in the system remain transparent or alternatively we can look at ^{13}C or ^{2}H or ^{15}N nuclei. Wide line apparatus[42], high resolution apparatus, and various pulse techniques[43] have

Table I

Time scale of various techniques

Physical technique	Time scale
X-ray diffraction	long time
NMR	10^{-6} to 10^{-8} sec.
ESR	10^{-10} to 10^{-12} sec.
IR	10^{-12} to 3 X 10^{-14} sec.
RAMAN	10^{-12} to 3 X 10^{-14} sec.

been employed in this study. Relaxation properties such as spin-lattice relaxation (T_1) and spin-spin relaxation (T_2) times of particular nuclei can be determined.

e) Electron paramagnetic resonance spectroscopy: Since most biological systems are diamagnetic, paramagnetic labels have to be incorporated into the systems in order to use this technique. Spin labels like nitroxide free radicals or the specially synthesized nitroxide stearate label have been used to prove structural and kinetic properties of biological membranes and pure phospholipid bilayer membranes[44-46]. That the biological membranes do contain a phospholipid bilayer was also proved by this technique[47]. The presence of a flexibility gradient in fatty acid chains of biological membranes was also proved by this technique. In this technique information about the organization of the membrane structure is inferred indirectly from its interaction with the probe. There are obvious limitations on the structural information the probe can provide. This is due to the fact that we do not usually know precisely where the probe is localized in the membrane. This technique gave information on the degree of fluidity of the hydrocarbon chain of the lipid bilayer. It was found that the hydrophobic regions of phospholipid bilayers and membranes become more and more fluid as one moves towards the terminal methyl group.

Various other physical techniques[48, 49] have also been used as probes of membranes and membrane models. Actually the results from these experiments enable one to construct more precise models for membranes and also to understand various biological functions such as active transport.

Infrared and Raman spectroscopy as tools:

The one important difference between the models, suggested for the arrangement of protein and lipid in biomembranes, is the degree to which lipid bilayers with ordered fatty acid chains, play a role in the structure. All the forementioned techniques aim at probing the molecular organization but are essentially relatively long time averaging techniques. Infrared and Raman spectroscopy, having a time scale 10^{-12} to 10^{-14} second, can be used to study fast molecular motions. It has been speculated that increased knowledge about mobility and organization of these molecules will lead to an understanding of the membrane properties. To this end, in this thesis infrared and Raman spectroscopy are employed for studying membrane models[50-52]. These studies indicate that study of molecular vibrations can be a sensitive probe of order in fatty acid chains of phospholipids. The number of bands observed, the relative intensities of the bands, and the band widths, all yield information on this subject. The ability to probe these bilayer

systems without introducing any chemical perturbation, using small amounts of sample (about 8nl), with simultaneous examination of all parts of the phospholipids, make infrared and Raman spectroscopy important methods for studies of biomembranes.

Results are presented on neutral lipid-water gels and mixed lipid gels. Infrared studies have also been done on the influence of anionic phospholipid on neutral lipid-water gels, as well as on a protein-lipid complex.

EXPERIMENTAL TECHNIQUES

A. Raman Instrument: Raman spectra were taken with a Spex Industries Model 1401. The instrument uses a double monochromator system as shown in figure 6. The light source was a Spectra-Physics Model 125 helium-neon laser. The exciting line in this system is 632.8 nm. The power of the laser beam at the source is about 80 mw but only about 50 mw power at the sample. This power was found to be quite adequate to get satisfactory spectra of lipid gels. The detection system of the instrument used the photon counting technique. Spectra were run at about 5 cm^{-1} spectral slit width. A Corning 2-62 filter was used to eliminate a Lyman ghost at 2902 cm^{-1}.

This laser was found to be extremely stable with usual short term peak-to-peak fluctuation of less than 1% as claimed by the manufacturer. There are several non-lasing helium and neon lines emitted. These are of lower intensity than the laser line and are eliminated by use of a spike filter.

The Raman instrument used to study the lipid-water gels employs a transverse viewing optical system suggested by Landon and Freeman[53]. This is shown in figure 7. In this system the focussed beam passes through the clear sample and can be reflected back again for another pass. Since the exit of the laser is itself a mirror, further reflection to the sample is achieved, thus enhancing Raman intensity over a single pass.

Figure 6

Double monochromator system of Raman instrument Spex model 1401.

S_1, S_2, S_3 Slits

M_1, M_2, M_5, M_6 Concave mirrors

M_3, M_4 Plain mirrors

G_1, G_2 Gratings

PM Photomultiplier

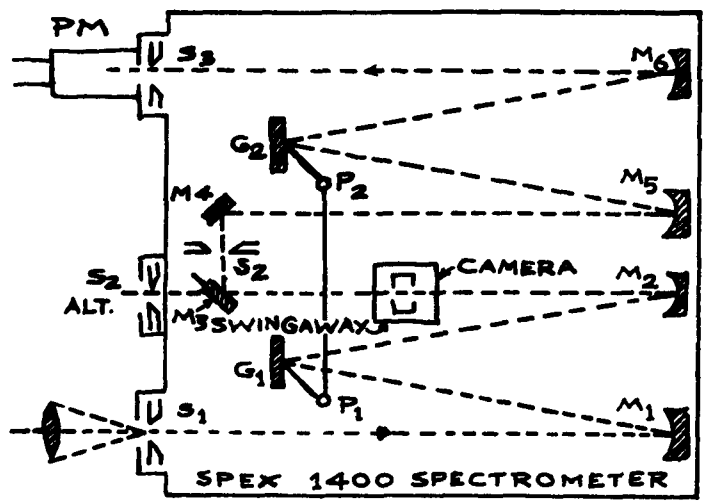

PM
S3
M6
G2
P2
M5
M4
S2
S2
CAMERA
M2
ALT.
M3 SWINGAWAY
G1
P1
M1
S1
SPEX 1400 SPECTROMETER

Figure 7

Transverse viewing optical system

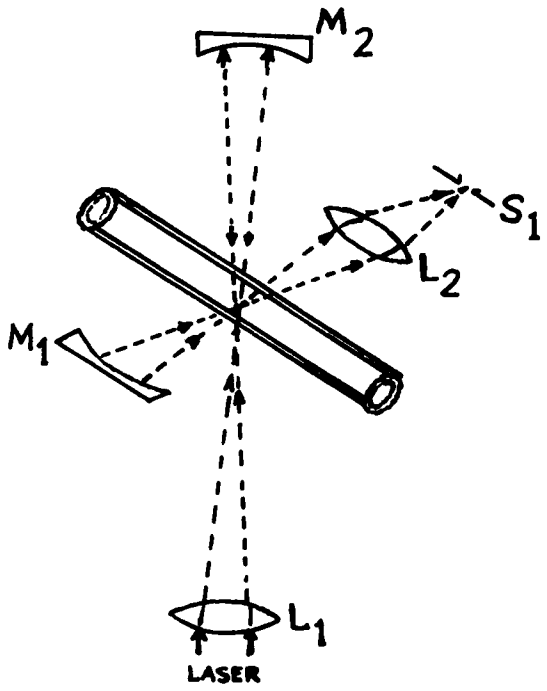
L_1 laser entrance lens

M_1 side condensing mirror situated perpendicular to the incoming laser beam

M_2 condensing top mirror

L_2 exit lens

S_1 slit to the spectrograph


M2
S1
L2
M1
L1
LASER

In conjunction with the instrument heating and cooling cells were used as accessories. The cross sectional diagram of the heating cell and base are as shown in figure 8. The heating cell was machined out of a cylindrical block of aluminum. The base of the heating cell was made out of a Teflon block so that heat would not be conducted into the base of the sample assembly. The heating and cooling cells were designed and constructed in our laboratory as added accessories to the Spex model 1401.

The temperature was measured by a copper-constantan thermocouple with the help of a potentiometer. The compensation for room temperature was done by immersing another junction of the thermocouple in a solid ice-water mixture.

The cooling below ambient was achieved by flushing the sample chamber by precooled nitrogen gas. Before flushing with precooled nitrogen, the entire sample chamber was flushed with dry nitrogen to preclude moisture in the chamber. If the moisture was in the chamber, the condensation of moisture over the sample at temperatures below ambient prevented the obtaining of good spectra of the sample. The block diagram for that assembly is as shown in figure 9.

Samples were generally contained in a capillary tube of diameter 0.5 to 1 mm and length of about 8 cm. The ends are sealed by a microburner.

B. Infrared Spectrometer: Infrared spectra were obtained

on a Perkin Elmer Model 521 dual grating spectrometer with a range of 4000 cm^{-1} to 250 cm^{-1}. The instrument was run at a spectral slit width of about 1 cm^{-1}. The spectra were recorded in chart paper whose ordinate represents transmittance (percent) and abscissa is linear in wave number. The sample was a thin film about 10 μ thick, held between IRtran-2 plates.

C. Differential Thermal Analyser: The DuPont 900 differential thermal analyser was used for the study of lipid-water gels. The samples are weighed and sealed in aluminum containers. The reference taken was glass powder. Samples were studied in the range from -20° to 80°.

D. Optical Method: Lipid - water gels were also studied by a Mettler FP2 melting point apparatus. With this instrument the light scattered by the sample was recorded with respect to temperature . The block diagram for this system is given in figure 10. The sample was contained in a capillary tube.

The lipid-water gels were also examined through a polarizing microscope with a provision to heat the sample. The pictures of the sample at different temperatures were also recorded by using a Polaroid camera loaded with instant color print film.

E. Barnes Variable Temperature Chamber: Model VTC-104 Barnes variable temperature chamber was used as an additional accessory with the infrared spectrometer. Temper-

Figure 8

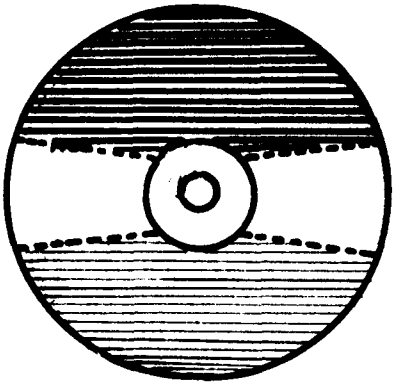
A cross sectional view of heating cell machined out of cylindrical block of aluminum

B cross sectional view of the base of the heating cell machined out of a cylindrical Teflon block

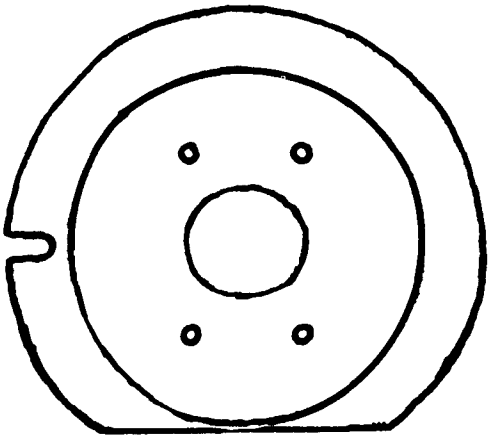
C cross sectional view of A at the centre

D another cross sectional view of B

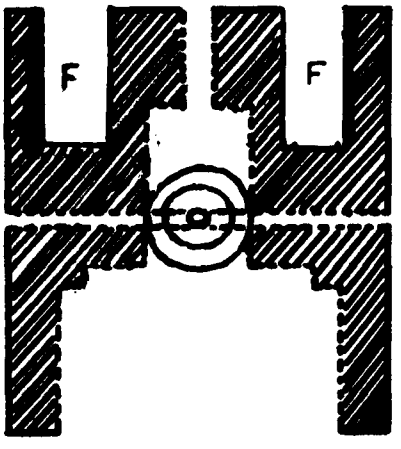
F cylindrical cavity for heating element



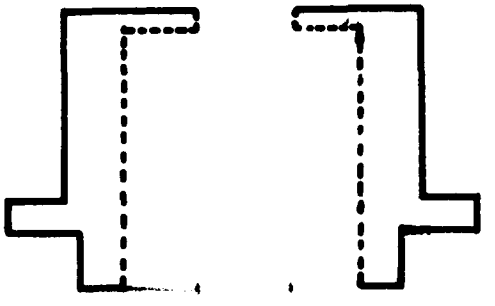
C



D



A



B

Figure 9

Block diagram for cooling cell

A: N_2 gas cylinder

B: Acetone dry ice mixture

C: Copper coils immersed in liquid nitrogen

D: Sample assembly

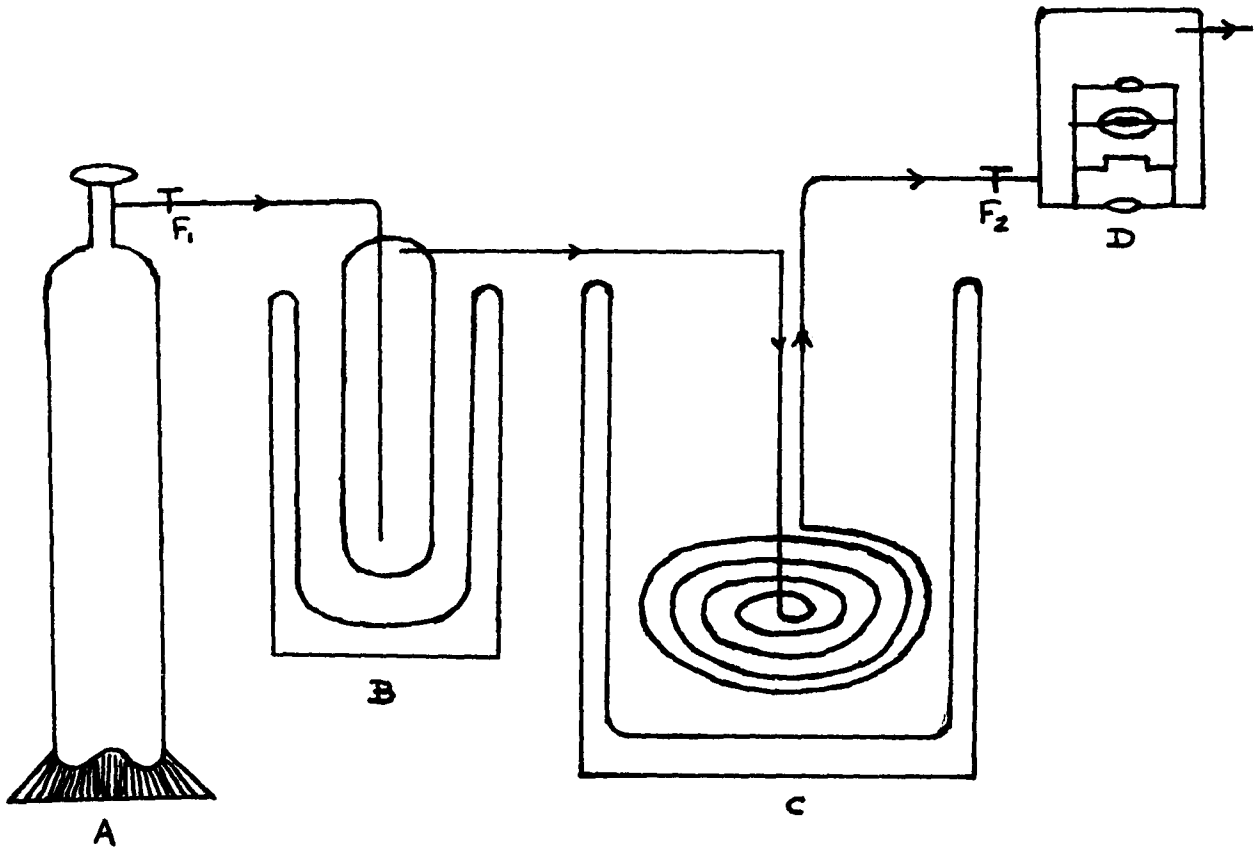

F1
F2
D
B
C
A

Figure 10

Block diagram of Mettler FP2 melting point apparatus.

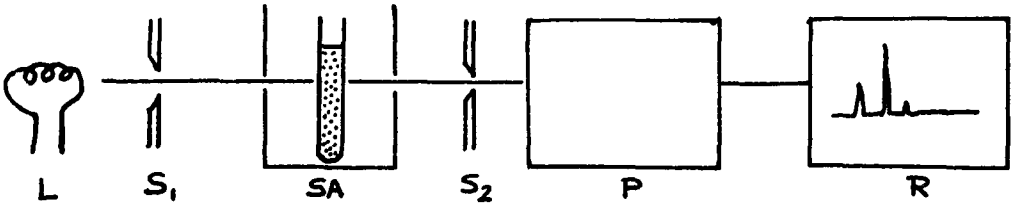
L: light source

S_1 and S_2: slits

SA: sample assembly

P: photocell

R: recorder


L
S1
SA
S2
P
R

ature inside the chamber was measured by the use of a built-in thermocouple, and a potentiometer. The sample was equilibrated for approximately 20 minutes for each temperature change before the spectrum was taken. The chamber was also flushed with dry nitrogen and sealed tight. For temperatures below ambient the infrared instrument was flushed with dry air in order to prevent condensation of water over KBr windows.

F. Sonifier: A Sonifier cell disruptor from Heat Systems Ultrasonic Model W 140 was used to prepare lipid-water gels. Weighed amount of lipid was transferred to a broad tip of the sonifier. A measured amount of distilled water was also transferred over the sample. This mixture was sonified for about 5 seconds. The gel sample was transferred to the IRtran plate to a uniform thickness. The thickness of the sample is varied by applying pressure over the plates. For Raman spectra the sample was withdrawn into the capillary tube with the help of a suction tube. The ends of the capillary tube were sealed by means of a micro blow torch.

Other Details: Some phospholipids used in the experiments, especially phosphatidyl serine, were obtained as a solution in benzene. The solution was taken in a capillary tube and zone evaporation was done by means of a vacuum system, but without much success. As a second attempt the evaporation was done over a hot plate by a careful technique.

The technique employed is as follows: The IRtran

plates are polished by using pure ethanol. A Kimwipe tissue paper was spread over a heater. The heater was put on for a few minutes until the temperature of the paper goes about 20° above ambient temperature. The heater was put off at this point. This takes a few minutes. The plate is then placed over the tissue. After a few seconds phosphatidyl serine solution was carefully pipetted out over the center of the plate by means of a microliter syringe. As soon as the solvent has evaporated a calculated amount of water was also pipetted out over the plate where the solid sample is located. A blower with cold air is used just 6" above the plate in horizontal direction. As soon as the water is added to the sample the second plate is placed over the first. By this time the temperature of the system comes to near ambient temperature. The plates are then pressed together and slight rotation on the plates makes the sample and water mix thoroughly. A thin film of the sample mixture was enclosed between the plates. The plates were now ready for mounting in IR cell frames for investgatigation. This was found to be very successful.

It was also found that lipid-water gels can be made by mixing weighed amount of lipid and water by means of micro mortar and pestle and then transferring the mixture to the IRtran plates, in which the mixture is sandwiched and heated above ambient temperature.

CHEMICALS

Phospholipids: All phospholipids used were chromatographically pure (tlc).

A Phosphatidyl Ethanolamine: This was supplied by Pierce Chemical Company (bacterial, chromatographically pure), a white powder which darkens at 170°. It was primarily a mixture of palmitic and oleic acid esters, with small amounts of stearic and linoleic acid chains also present. The average molecular weight of this material is 725.

B Lysophosphatidyl Ethanolamine: This was prepared from sample A and has the same characteristics. The average molecular weight of this material is 463.

C Lecithin: This was obtained from Mann Research and was tlc pure L-2,3 dihexadecyl glycerine-1-phosphoryl choline. This is also a white powder. The molecular weight is 761.

D Lysolecithin: This was also pure hexadecyl compound. The molecular weight is 508.

E Phosphatidyl serine: (Bovine): This was also tlc pure and obtained from Supelco as a solution in benzene (25 mg/ml). The molecular weight was approximately 788. and %P = 3.93. It probably contains a high degree of unsaturated fatty acids.

F Cytochrome C: This was obtained, as red flaky powder from Sigma Chemical Company. This Cytochrome C type VI was prepared from Horse heart by acetic acid extraction without the use of trichloracetic acid. The molecular weight was reported as 12,384. Assay was reported as 95% corrected for 6.4% water when prepared.

Other chemicals such as sodium dithionite used were common Reagent grade compounds.

EXPERIMENTS AND RESULTS

Ia Infrared spectra of neutral lipid-water gels

One of the most important problems in membrane biology is the physical and chemical organization of phospholipid interactions. To this end many physico-chemical techniques have been used with membrane model systems. The first and foremost of those models chosen is the lipid-water gel. It is well-known that anhydrous phospholipids can exist in liquid crystalline forms. Chapman[54] examined the infrared spectrum of DL-α-dipalmitoyl phosphatidyl ethanolamine in a KBr disc as a function of temperature from -186 to +140°. The spectrum at low temperature resembled that of a crystalline solid, showing splitting of bands in various spectral regions. This splitting disappeared as the temperature was raised and the spectrum resembled that of a fluid rather than a solid phase. Figure 11 shows the infrared spectrum of anhydrous phospholipid heated to various temperatures.

Recent studies [32, 55-57] on many phospholipid-water systems by X-ray diffraction and electron microscopy indicate that these systems exist in a number of well defined mesomorphic phases (lamellar, hexagonal, cubic). Figure 12 shows a schematic diagram of these lipid-water phases. In this figure the hydrophilic group is represented by a dot, the paraffin chains by a wiggle.

Figure 11

Infrared spectra of 2,3-dilauroyl-DL-phosphatidyl ethanolamine at different temperature . (from reference 54)

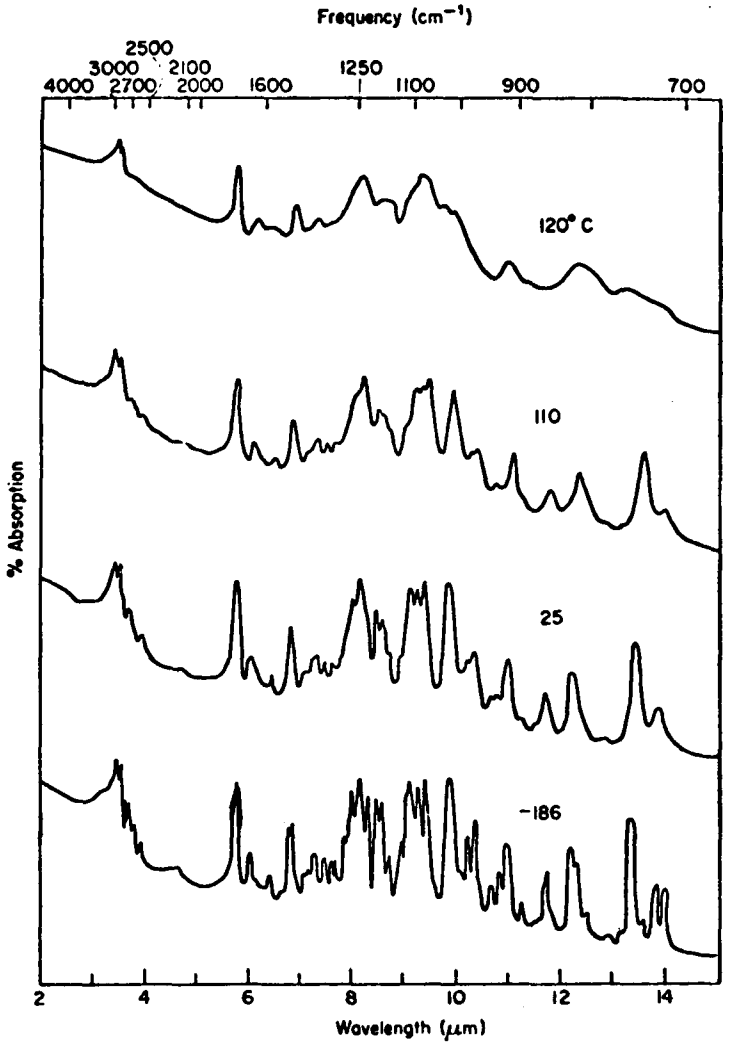

Frequency (cm⁻¹)
2500
3000
2100
4000
2700
2000
1600
1250
1100
900
700
120° C
110
25
-186
% Absorption
2
4
6
8
10
12
14
Wavelength (μm)

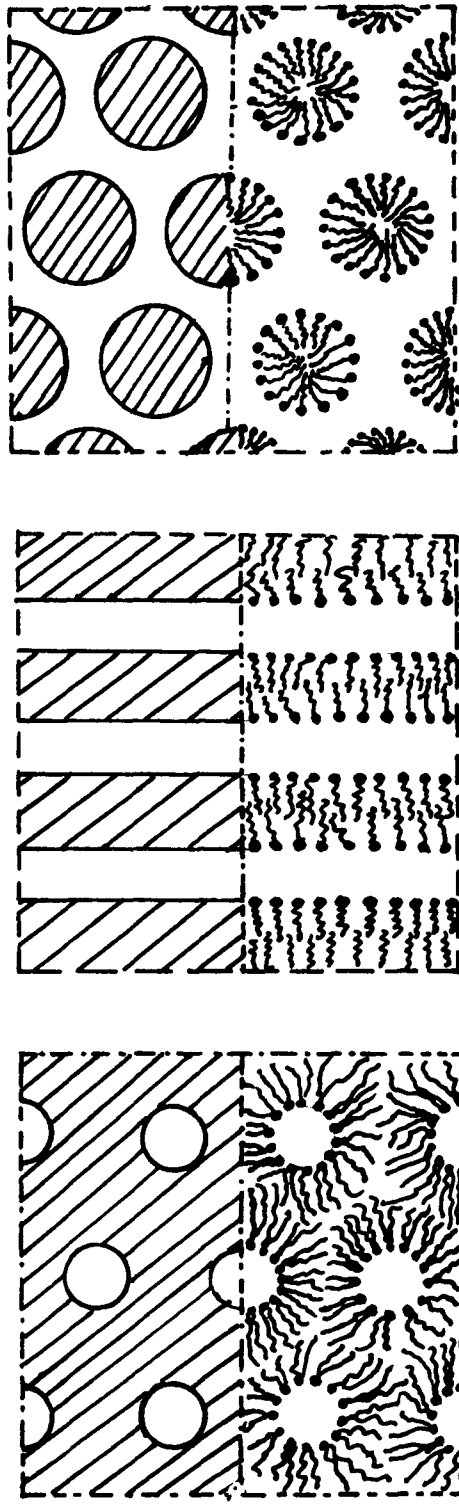
Figure 12

Structure of some high-temperature phases of lipid-water (gel) system (from reference 57).

H_1 Hexagonal 1 - is a hexagonal array of indefinite; the hydrocarbon chains fill the interiorof the cylinders, water outside.

L Lamellar - is an alternate sequence of planar layers of lipid and water.

H_2 Hexagonal 2 - is similar to hexagonal 1, but water is in the interior of the cylinders, the hydrocarbon chains fill the gap between the cylinders.


H_1
L
H_2

Lipids by themselves do not disperse in water because of the presence of non-polar hydrocarbon chains in them. It was found that in order to form a lipid gel with water, dispersion of lipid in water can be accomplished by sonication or sometimes by heating. When sonication was used with a lipid-water mixture, the resulting gel was found to exist in one well-defined mesomorphic phase by X-ray crystallography. In the absence of sonication, lipid and water exist as two distinguishable phases rather than as one single gel phase.

Lipid (lysophosphatidyl ethanolamine) suspended in 20% water was sandwiched between two well polished IRtran plates and the infrared spectrum of the lipid-water mixture before sonication is given in figure 13. The same mixture was transferred to the flat tip of the sonicator and the sonication was carried out for five seconds. The resulting lipid-water gel was then transferred to between two well cleaned and polished IRtran plates and the infrared spectrum of the sandwiched gel is as shown in figure 14.

The differences between these spectra are dramatic. In the case of unheated and unsonicated lipid-water mixture there are a number of sharp bands in the region 2000cm^{-1} to 1000cm^{-1}, whereas the sonicated sample presents not only fewer but also broader bands in the same region. However, both these spectra contain a sharp band in the region 1470 cm^{-1}. The spectrum shown in figure 13 is identical with that

Figure 13

Infrared spectrum of lipid suspended in water, before sonication. Between 2000 and 1000 cm^{-1}

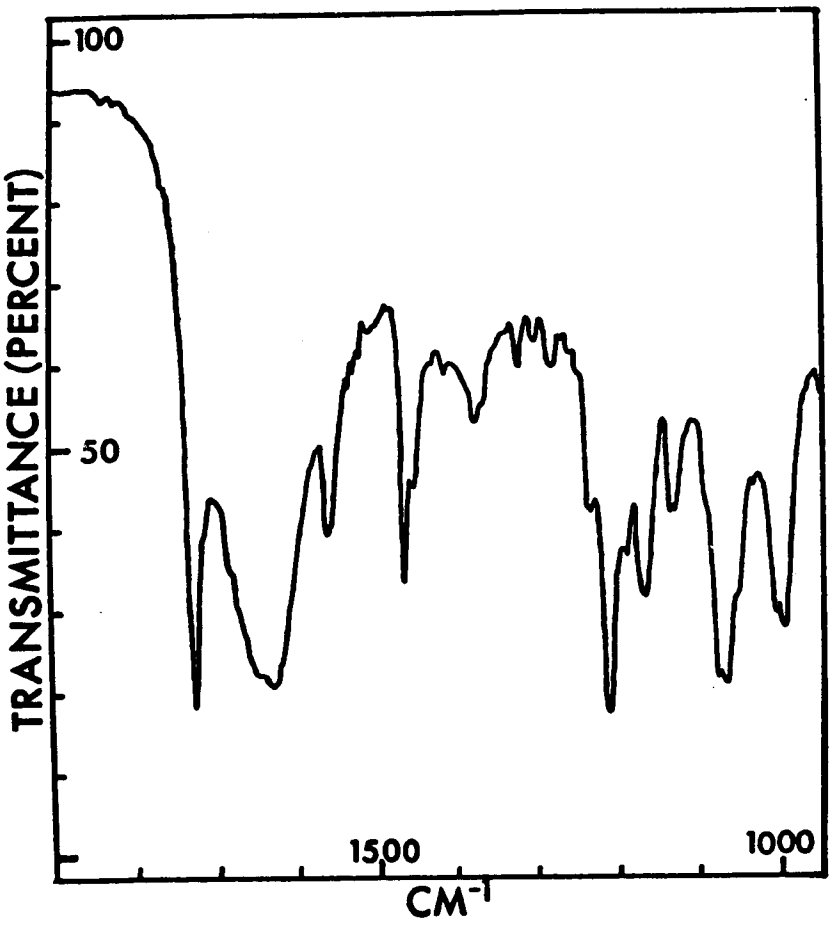
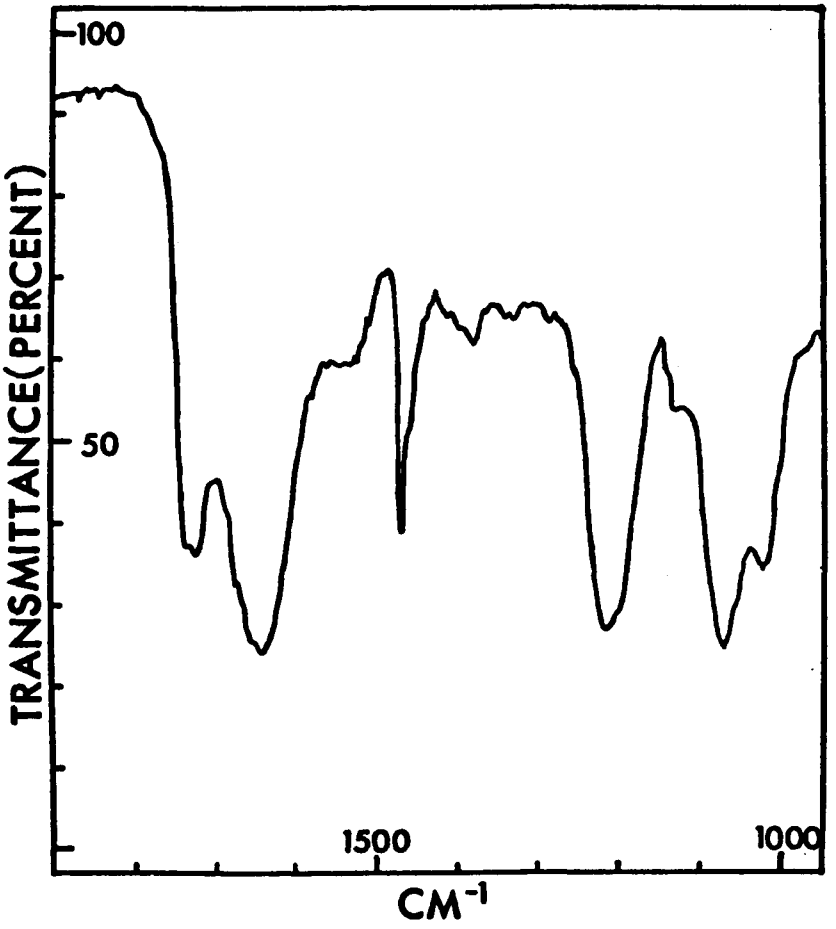

100
50
TRANSMITTANCE (PERCENT)
1500
1000
CM⁻¹

Figure 14

Infrared spectrum of lipid gel, after sonication.

Between 2000 and 1000 cm^{-1}


100
50
TRANSMITTANCE(PERCENT)
1500
1000
CM-1

seen in a sample of the pure solid in Nujol with the addition of the strong absorption due to water near 1640 cm^{-1}. One notes that this spectrum contains a number of sharp, well-defined bands, characteristic of the infrared spectra of pure solids. The differences between these two spectra (figures 13 and 14) may be summarized as follows: (1) There are fewer distinct bands in the spectrum of the gel than in that of the solid, (2) nearly all bands in the gel appear much broader than those of the solid. An exception to (2) is the band at 1470 cm^{-1}. This methylene deformation vibration band is quite as sharp in the gel as it is in the solid.

The spectra in figures 13 and 14 are characteristic of phospholipid-water systems. Identical results have been observed with phosphatidyl ethanolamine (PEA), lecithin (PC), lysolecithin (LL), as well as lysophosphatidyl ethanolamine (LPEA) gels. The spectra of PEA-water gel, PC-water gel and LL-water gel are given in figures 15, 16, and 17 respectively. The differences between these spectra are minor. Differences reflect the length of the hydrocarbon chain and their number present in the lipid, as well as differences in the number of methyl groups on the polar-head group. Table II gives the appropriate band assignments of the spectra of these lipid gels.

When these gels are heated, the spectra remain unchanged, with the exception of the band at 1470 cm^{-1}. Its peak intensity is observed to decrease relative to that of the

Table II

Appropriate band assignments of the spectra of lipid gel

Wavenumber (cm^{-1})	Group vibration	strength
1740	C=O	strong
1555	NH_3 deformation	medium
1470±5	$-CH_2$ scissoring	strong
1460	$-CH_3$ asymmetric deformation	shoulder
1420±5	$-O-CH_2$ scissoring	weak
1245±5	P=O	strong
1200±5	C-O	weak
1085±5	$P-O^-$	strong
1060±5	(P)-O-C-	strong

Assembled from

References 86 and 87.

Figure 15

Infrared spectrum of PEA-water gel between 2000 and 1000 cm^{-1}.

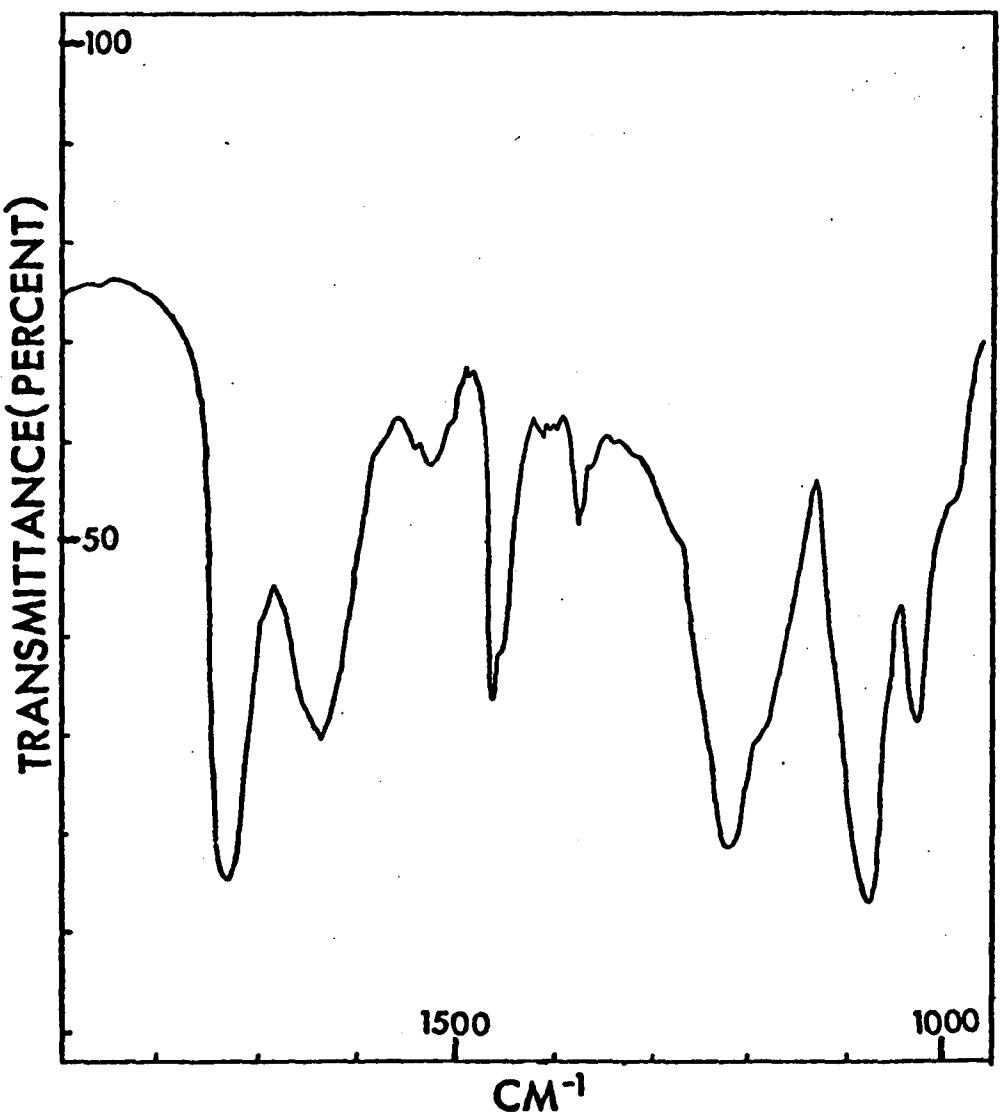

100
50
TRANSMITTANCE(PERCENT)
1500
1000
CM-1

Figure 16

Infrared spectrum of lecithin gel between 2000 and 1000 cm^{-1}.

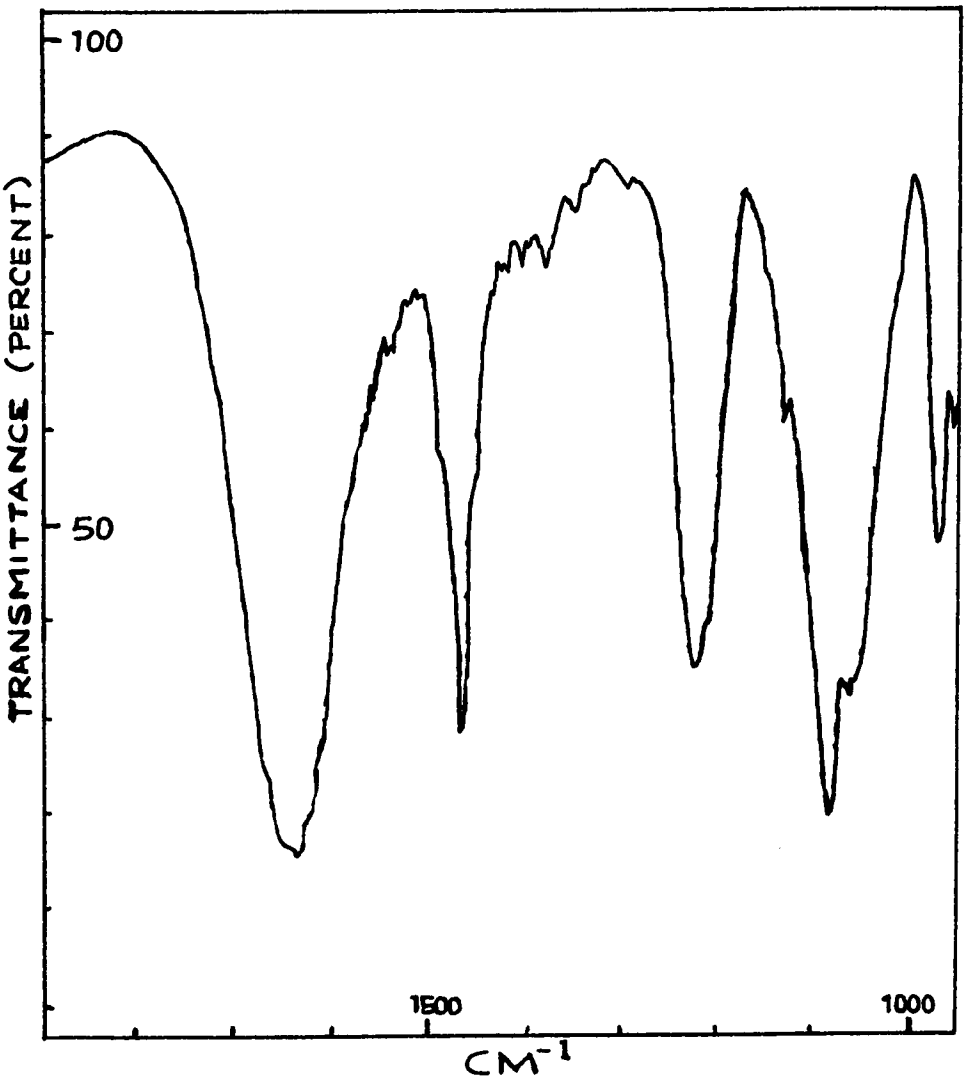
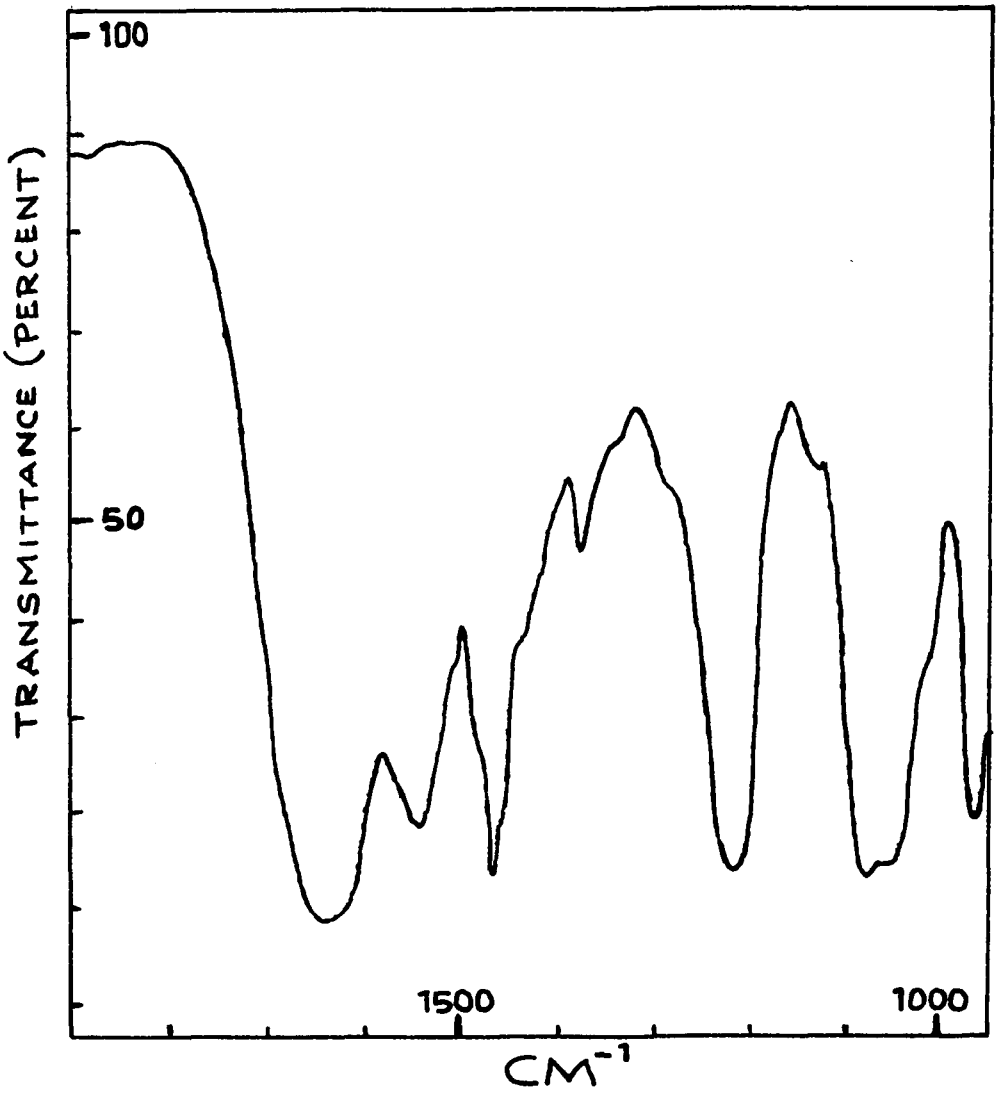

100
50
TRANSMITTANCE (PERCENT)
1500
1000
CM⁻¹

Figure 17

Infrared spectrum of LL gel between 2000 and 1000 cm^{-1}.


100
50
TRANSMITTANCE (PERCENT)
1500
1000
CM⁻¹

other bands in the spectrum. Plots of this relative absorbance vs. temperature are shown in figure 18 (A-D). These plots reveal at least one phase transition, and in some cases, several distinct transitions, for each system. All points in these plots represent equilibrium measurements, averaged for several runs. In these plots the computed uncertainty in a given absorbance ratio is ± .005, using propagation of errors theory, and in a given temperature, ± 1°. Around 70 to 80°, in some cases over 80°, the sample appears to break down and many spectral changes, probably including loss of water, are observed.

It will be of great interest to know why the relative absorbance of the band at 1470 cm^{-1} with respect to another in the spectrum varies as the temperature of the gel is varied.

One can hypothesize two mechanisms for the change in intensity at 1470 cm^{-1}. The first reason may be that there is an overall change in average dipole derivative associated with this vibration as the temperature is raised, which would cause the intensity of the band to decrease as the temperature in increased. The second reason may be that the intensity shifts towards the wing, thus causing the peak intensity of the band at 1470 cm^{-1} to alter as the temperature of the sample in increased. The first thing to glean from these spectra is to evaluate what happens to this band. Two measurements were done on these spectra.

Figure 18

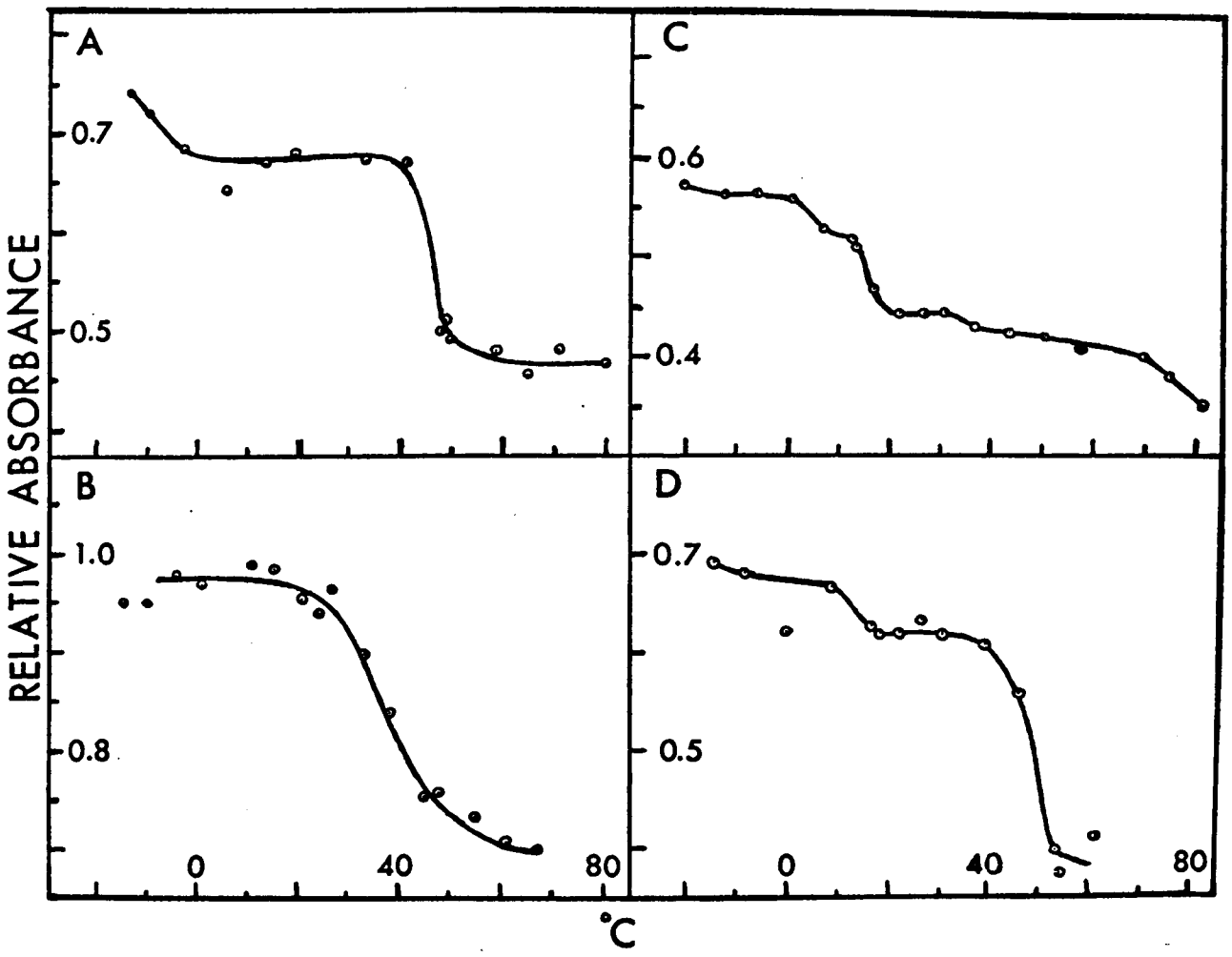
Relative absorbance *vs.* Temperature graph of

A. Lecithin gel

B. LL gel

C. PEA gel

D. LPEA gel


A
B
C
D
0.7
0.5
1.0
0.8
0.6
0.4
0.7
0.5
0
40
80
0
40
80
RELATIVE ABSORBANCE
°C

For this purpose, the spectra obtained by using phosphatidyl choline lipid-water gel sample were chosen as representative.

With a planimeter the areas under the peak at 1470 cm^{-1} and 1220 cm^{-1} were measured. In addition the half band width of these two bands were measured. A plot of the ratio of the areas against the temperature of the sample produces a straight line and a plot of the ratio of the half band width against the temperature of the sample produces a sigmoidal type of curve. These plots are shown in figures 19a and 19b respectively. It is clear from Tables III and IV that the area and the half band width of the band at 1220 cm^{-1} are remaining constant throughout the experimental temperature range. While the area of the band at 1470 cm^{-1} is remaining constant, the half band width is found to increase with increasing temperature.

Ib Raman spectra of neutral lipid-water gels

Raman spectra (100-4000 cm^{-1}) of the four neutral phospholipid-water gels (20% water) have been measured as a function of temperature. Figure 20 shows the Raman spectra of a lecithin-water gel between the region 500-3100 cm^{-1}. Infrared spectral measurements conceal certain features which are revealed by Raman spectra. The foremost among these arises from the intense infrared spectrum of water, which obscures the 3000 cm^{-1} region (C-H stretching) as well as the lower frequency region ($<$700 cm^{-1}).

Table III

Area measurements of bands at 1470 cm^{-1} and 1220 cm^{-1} with respect to the temperature of a lipid gel sample.

Lipid : L-2,3 dihexadecyl glycerine 1 phosphoryl choline.

Temperature	Area of		Relative area
C	A_1 band at 1470 cm^{-1}	A_2 band at 1220 cm^{-1}	
24	80	150	.53
26	78	148	.53
26.5	83	151	.55
28	80	146	.55
34.5	80	154	.52
41.5	83	153	.54
48	85	157	.54
49	83	151	.55
50	83	152	.55
59	84	154	.55
65	85	151	.56
71	87	159	.55
80	84	158	.53

Table IV

Temperature vs. half band width of lipid gel.

Lipid : L-2,3 dihexadecyl glycerine 1-phosphoryl choline

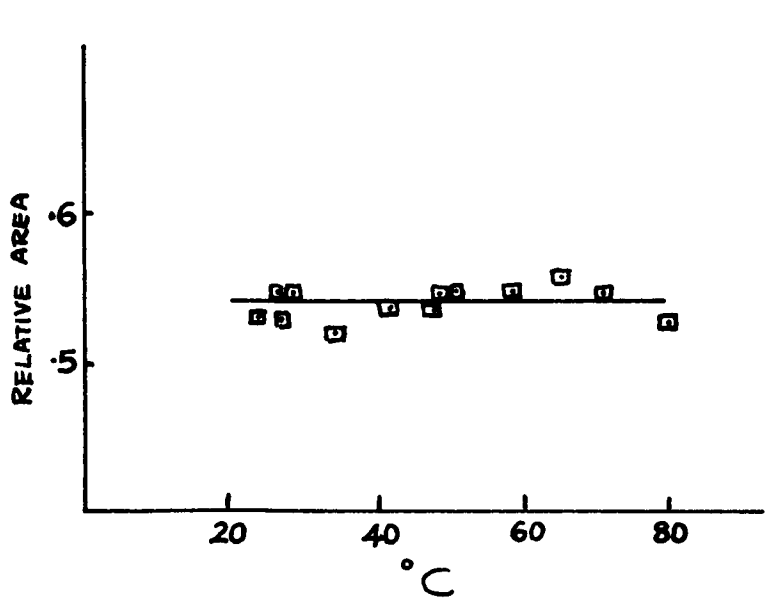
Temperature C	Half band width of band at 1470 cm^{-1} mm.	Half band width of band at 1220 cm^{-1} mm.
-13	4.25	10.0
- 9	4.50	10.0
- 2.5	4.70	10.0
6	4.75	10.0
13.5	4.75	10.0
19.5	4.75	10.0
24	4.75	10.0
26	4.75	10.0
28	4.75	10.0
34.5	5.50	10.0
41.5	5.50	10.0
49	7.25	10.0
50	7.50	10.0
59	7.60	10.0
65	7.75	10.0
71	7.75	10.0
80	7.75	10.0

Figure 19

A. Relative area vs. Temperature of lecithin gel

B. Half band width vs. Temperature of lecithin gel

Plotted from data given in Tables III and IV.


A
RELATIVE AREA
·6
·5
20
40
60
80
°C

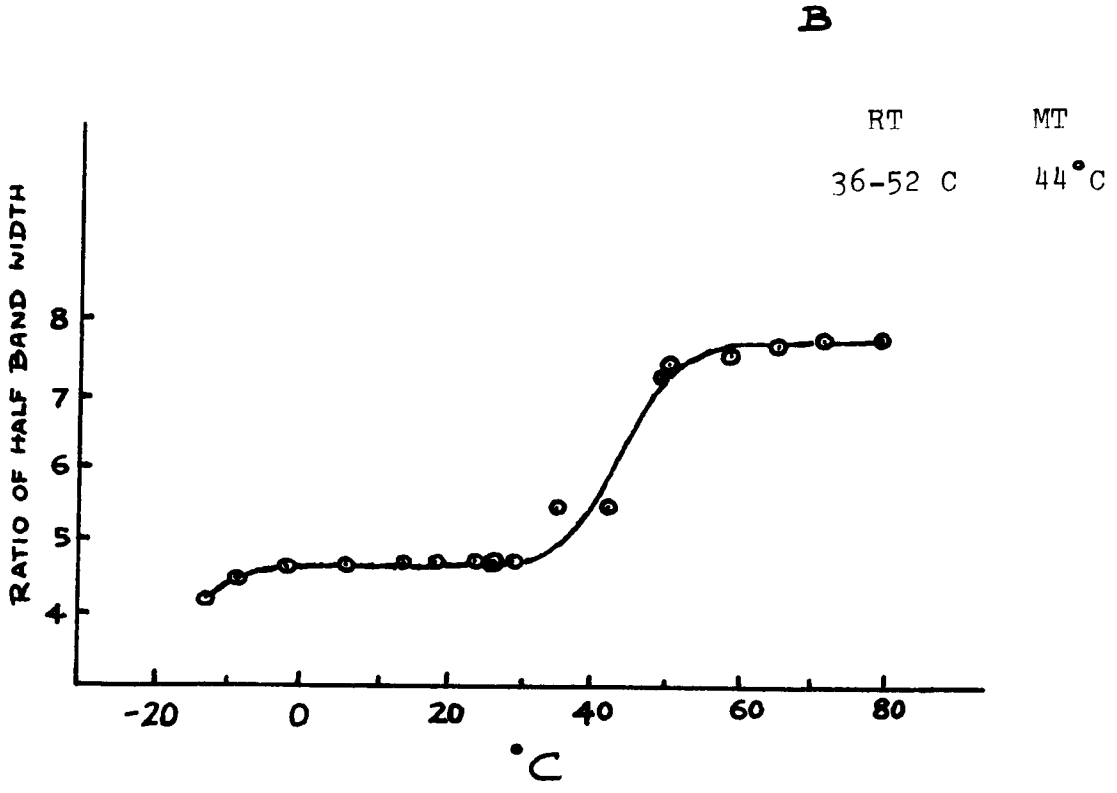

B
RT
MT
36-52 C
44°C
RATIO OF HALF BAND WIDTH
8
7
6
5
4
-20
0
20
40
60
80
°C

Figure 20

Raman spectrum of lipid gel (500 to 3100 cm^{-1}).

Computerized laser Raman

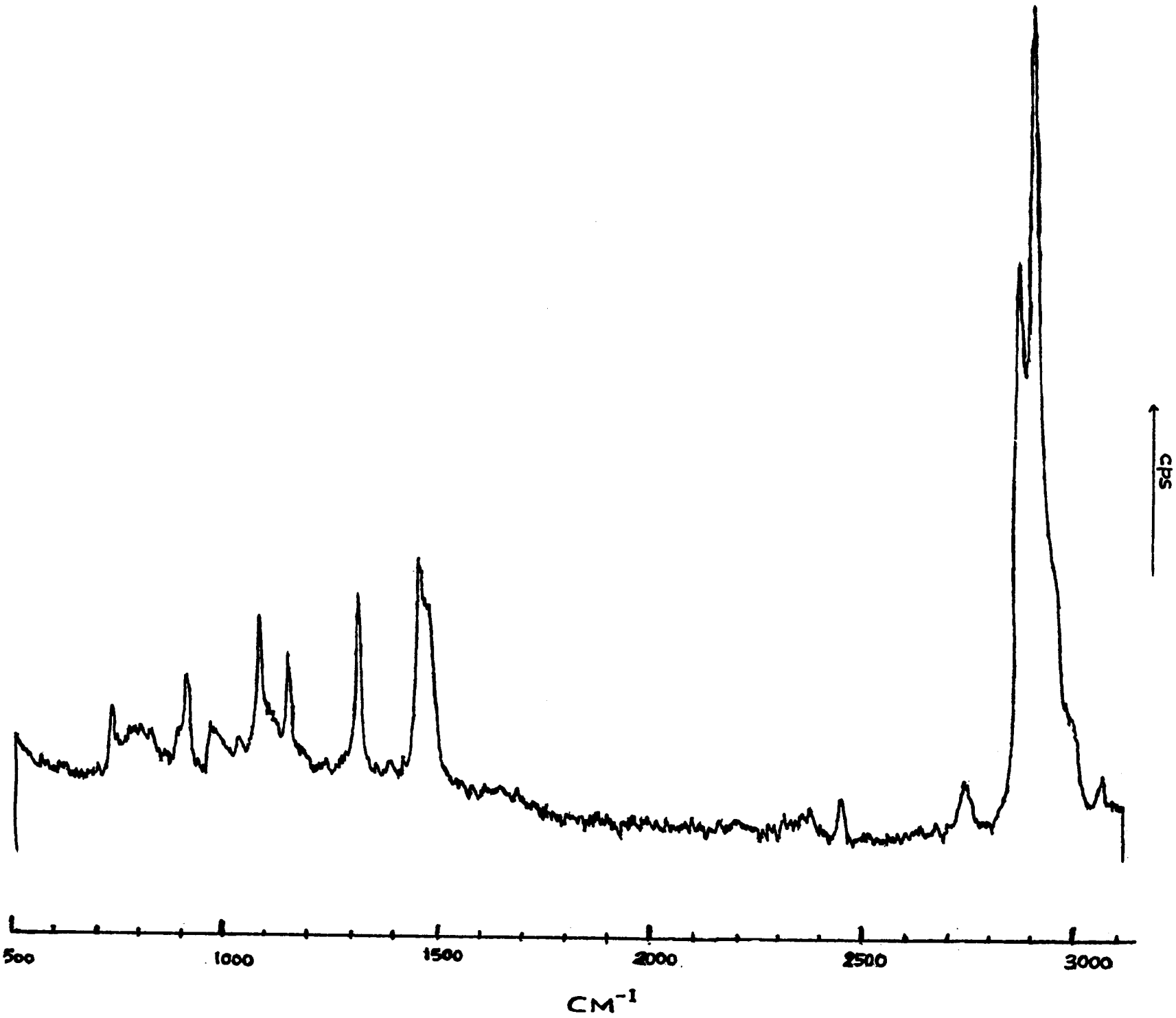
Laser used : Krypton

Exciting line : 530.9 nm

Lipid used : Dipalmitoyl lecithin

Number of points : 2600

Rate : 5 sec/cm^{-1}


cps
500
1000
1500
2000
2500
3000
CM-1

While the Raman spectrum of water is quite weak, C-C stretching vibrations appear relatively intense. These bands give rise to very weak infrared absorption. The complete Raman spectra for the phospholipid-water systems have been examined as a function of temperature. Again, as in the infrared spectra, many of the bands are insensitive to temperature variation and hence to the phase transition. For example, figure 21 shows the phosphate vibration near 700 cm^{-1} in a lecithin-water gel, which undergoes no significant change as the temperature is raised.

Changes do occur, however, in the spectral regions associated with the fatty acid chains. Figure 22 shows this for A) lecithin and B) lysolecithin gels respectively in the C-H stretching region. Identical changes to those shown for lecithin and lysolecithin gels are found in the spectra of phosphatidyl ethanolamine and lysophosphatidyl ethanolamine gels. The two intense bands at 2840 and 2875 cm^{-1} are symmetric and asymmetric vibrations of the methylene groups. As the temperature is raised, these bands change in relative intensity.

Raman spectra of normal and deuterated polyethylene in the solid and liquid states were obtained by Brown[58]. Figures 22 C and D show the Raman spectrum of solid and molten polyethylene redrawn from his paper.

Figure 21

Temperature dependence of Raman spectrum of a lecithin-water gel near 700 cm^{-1}.

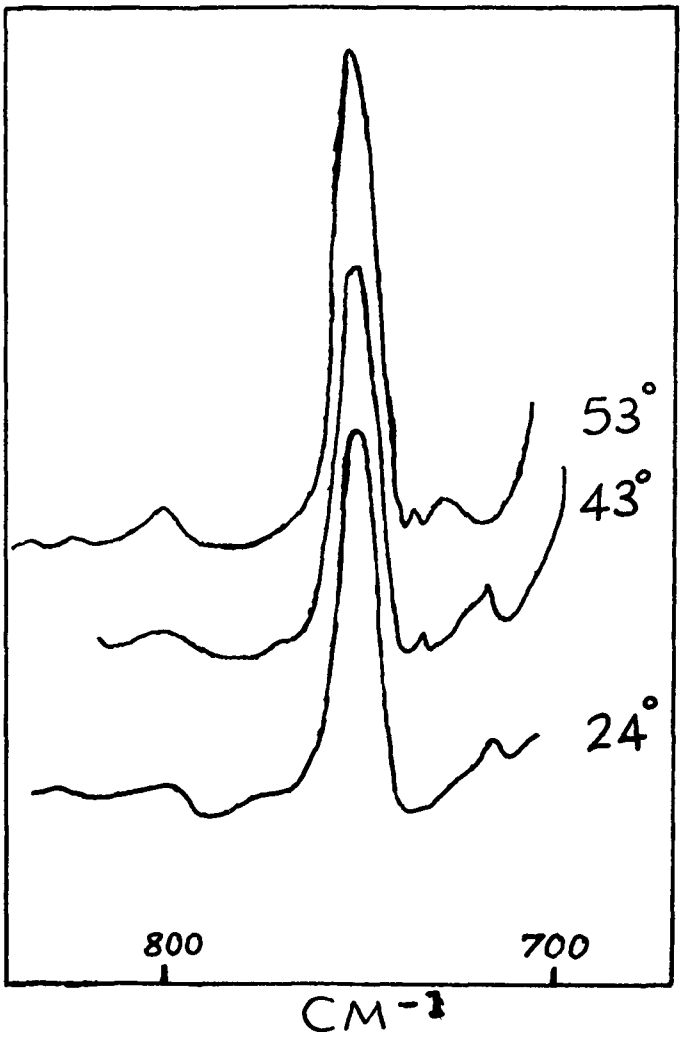

53°
43°
24°
800
700
CM-1

Figure 22

Temperature dependence of Raman spectrum of (A) lecithin-water and (B) lysolecithin-water gels in the C-H stretching region. (C) and (D) are spectra of solid and molten polyethylene, respectively, in the same region (redrawn from reference 58).

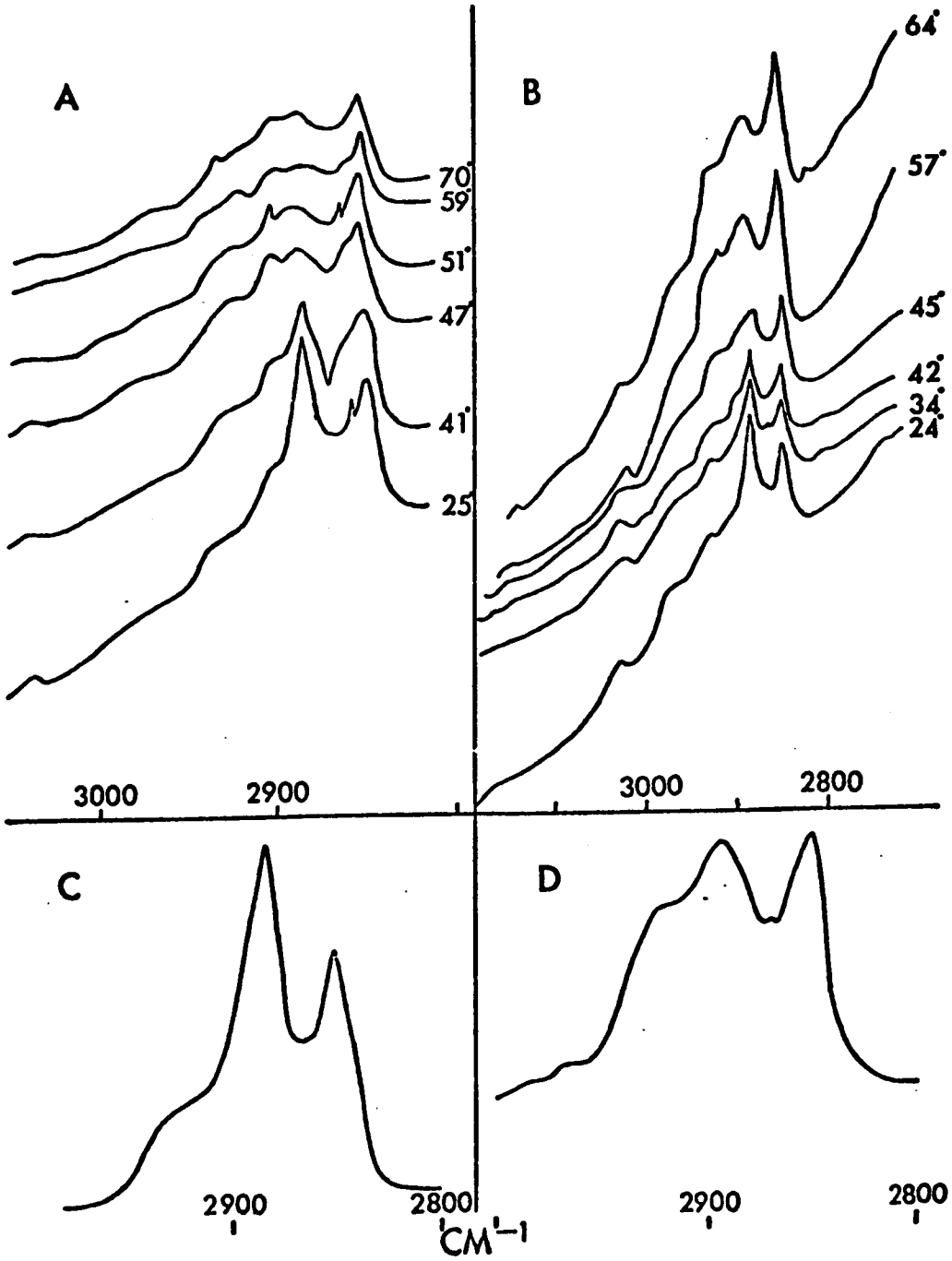
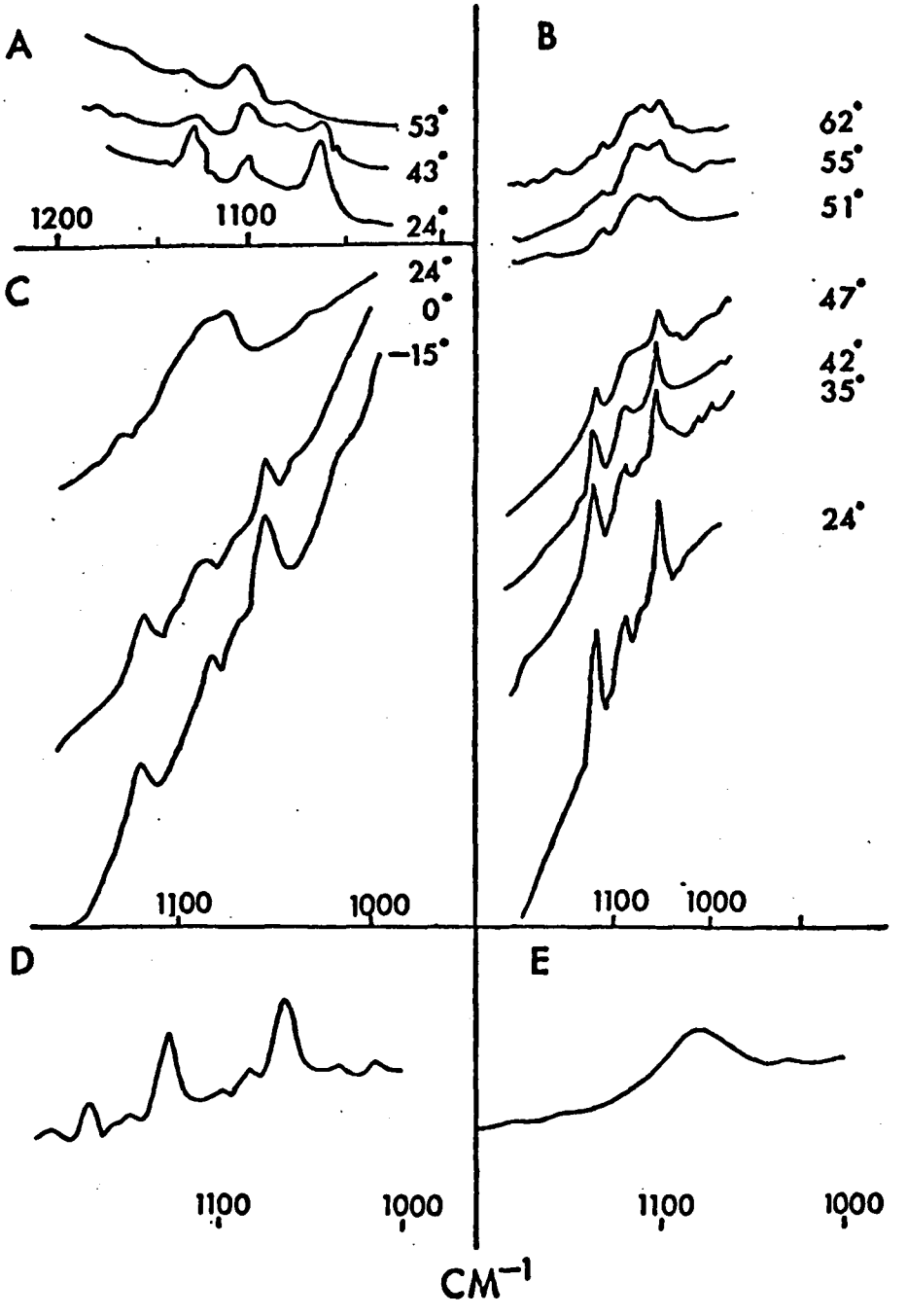

A
70°
59°
51°
47°
41°
25°
3000
2900
B
64°
57°
45°
42°
34°
24°
3000
2800
C
2900
2800
D
2900
2800
CM⁻¹

Figure 23

Temperature dependence of Raman spectrum of (A) lecithin-water, (B) lysolecithin-water, and (C) phosphatidyl ethanolamine-water gels in the C-C stretching region. (D) and (E) are solid and molten polyethylene, respectively (redrawn from reference 58).


A
53°
43°
24°
1200
1100
B
62°
55°
51°
C
24°
0°
−15°
1100
1000
47°
42°
35°
24°
1100
1000
D
1100
1000
E
1100
1000
CM⁻¹

The C-C stretching region also changes with temperature. This is illustrated for lecithin, lysolecithin and phosphatidyl ethanolamine gels in figures 23 A, B and C respectively. High quality spectra for lysophosphatidyl ethanolamine gel could not be obtained in this region because of high background scattering. It appears that three bands collapse to a broad, central maximum as the temperature is raised. The analogous spectra in solid and molten polyethylene, again redrawn from Brown's paper, have been included for comparison.

Many attempts were made to obtain spectra in the lattice vibration region. The spectra taken at various temperatures in this region do not show any appreciable changes. Attempts were also made to complement these spectra by far infrared Fourier transform spectroscopy of these lipid-water gels. However, the liquid water absorption from these gels made it impossible to obtain good spectra in this region, 20-400 cm^{-1}.

II Mixed lipids-water gels by infrared spectroscopic techniques

It is of interest to choose model systems close to the membrane systems existing in nature. Natural systems contain a mixture of phospholipids of different classes, with varying degrees of saturation and with different numbers of hydrocarbon chains. Mixed phospholipid systems have not been investigated thoroughly despite their importance in natural systems. The effect of the number of hydrocarbon chains on the phase behavior of mixed phospholipid systems may give a clue to the function of hydrocarbon chains in membrane systems. Studies[59, 60] of mixed lecithin systems have given some understanding of how molecules with different chains pack in the bimolecular lamellae in excess water and in monolayers.

For a mixture of distearoyl lecithin and dipalmitoyl lecithin, temperature composition diagram[59] showed a continuous series of solid solution formed below the boundary of crystal to liquid crystal phase transition. There was no evidence of compound formation or eutectic behavior. A mean molecular area vs. composition diagram for this mixture indicated cocrystallization and also found the ability to form mixed monolayers at 22° regardless of the state of the pure dipalmitoyl lecithin films. The same kind of studies were also performed for mixtures of dis-

tearoyl and dimyristoyl lecithins. Differential scanning calorimetry curves[60] for a series of equimolar mixtures of fully saturated lecithins with dioleoyl lecithins indicated monotectic behavior. These experiments were done with anhydrous lecithins as well as with hydrated lecithins.

The intermolecular chain mixing phenomena were proposed to be the reason for the broad chain melting transition observed with egg-yolk lecithin in water.

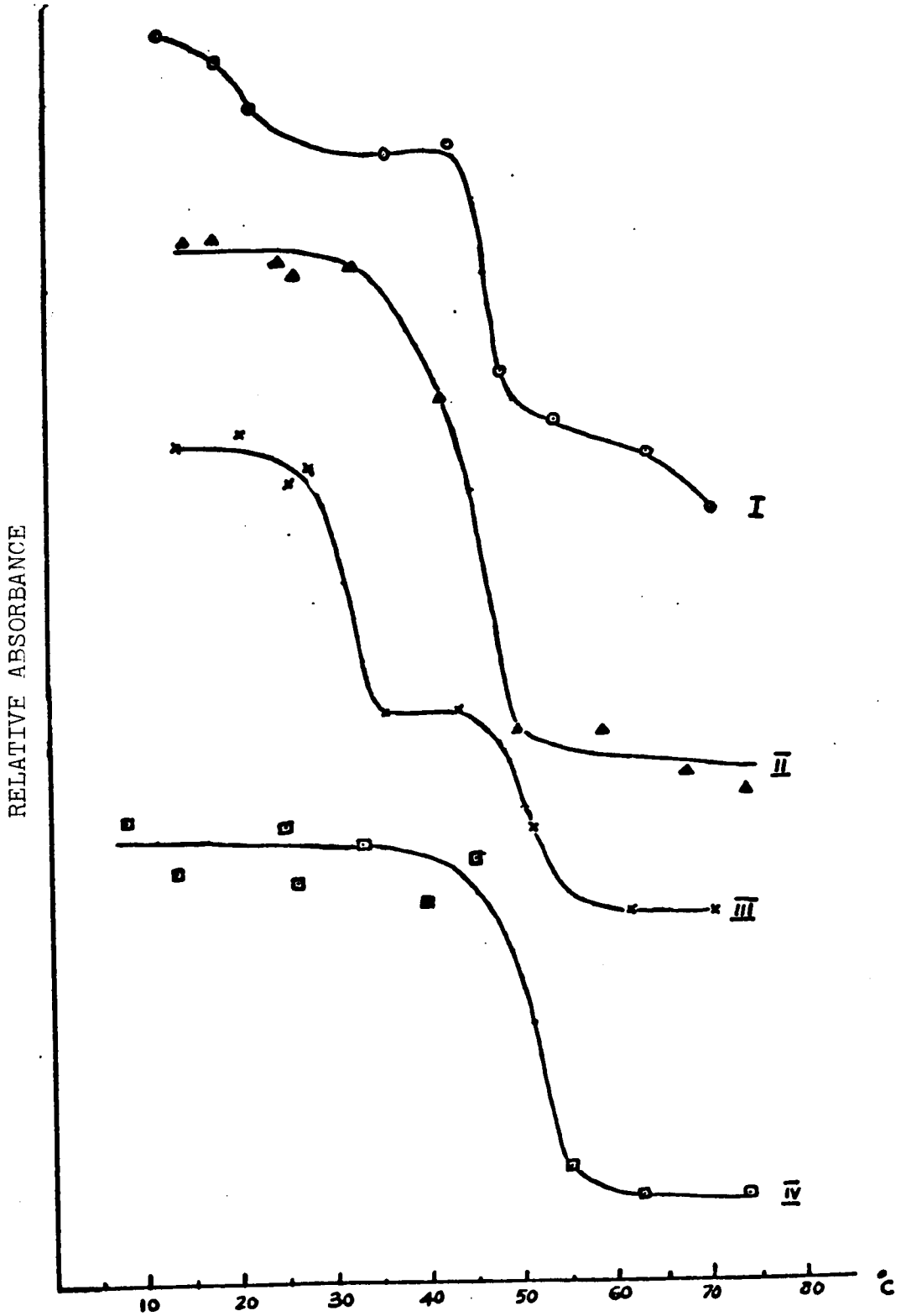
With these studies in mind a model system of mixed lipids (lysolecithin and phosphatidyl choline) were chosen. All the lipid gels were prepared by mixing weighed amounts of lysolecithin and phosphatidyl choline with 25% water. A brief sonication was of much help in preparing the gels. The gels were then transferred to IRtran plates. The sandwiched gels were placed in a variable temperature cell and their infrared spectra taken at different temperatures.

The relative absorbance of the 1470 cm^{-1} band with that of another band in the spectrum was plotted against temperature and is given in figure 24. The curves obtained are of sigmoidal shape characteristic of the phase transition.

The curve I represents a gel containing .96 X_{PC} (X_{PC} = mole fraction of phosphatidyl choline) and so on as indicated in figure 24. The gel with minimum X_{PC} studied

Figure 24

Change in absorbance (relative to another band in the spectrum) of infrared band at 1470 cm^{-1}, as a function of temperature, for mixtures of neutral lipids-water gels. Phosphatidyl choline-lysolecithin -water gels.


RELATIVE ABSORBANCE
I
II
III
IV
10
20
30
40
50
60
70
80
°C

contains .51 X_{PC}. All these gels contained approximately 20 to 25% water.

The analysis of the sigmoidal curves obtained in all these lipid gel systems requires an exact definition of transition temperature. Many of these curves are broad and some of them contain more than one transition. This makes it difficult to define one single transition temperature for all these curves. If the transition is of first order then there will be only one transition temperature; the onset temperature (OT), where the transition begins and the end temperature (ET), where the transition ends, become one and the same. For transitions other than first order (lambda transitions, etc.) it becomes necessary to analyze these sigmoidal curves by defining two quantities, namely (1) Range of Temperature (ET-OT=RT), the temperature range between the onset and the end of transition and (2) Middle transition temperature (MT). In practice there is a small pretransition and post transition effect. For such transitions it can be expected that the average of onset and end temperature would be equal to the mid temperature (MT). How these onset, mid and end of transition temperatures are determined, is shown in figure 25, for a typical sigmoidal type of curve obtained for these model systems. In forthcoming sections, the analysis of these curves will be done on the basis of onset temperature (OT), range of temperature (RT) or mid temperature (MT).

Figure 25

Estimation of onset, middle and end temperatures for a typical sigmoidal curve obtained for lipid gels by plotting relative absorbance vs. temperature.

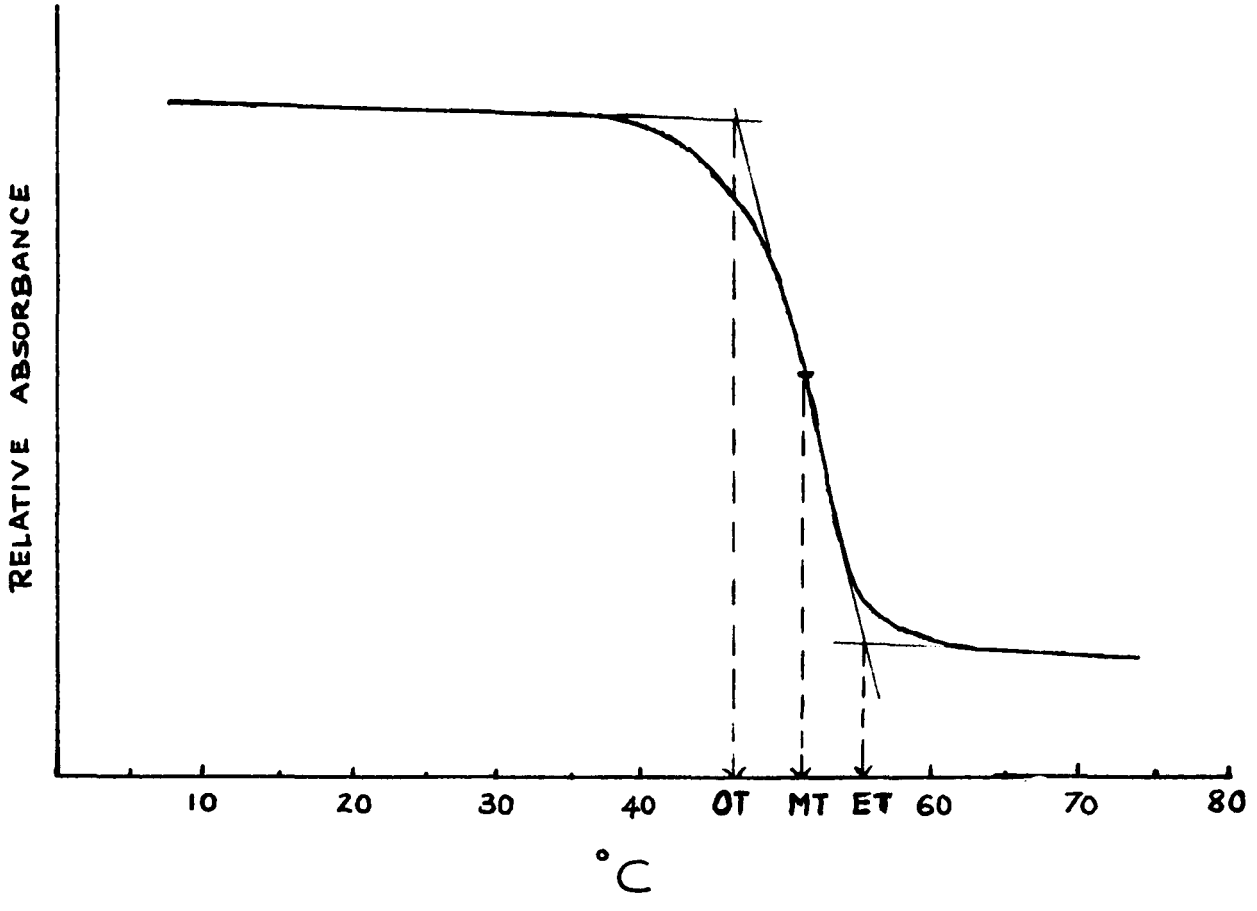

RELATIVE ABSORBANCE
10
20
30
40
OT
MT
ET
60
70
80
°C

Table V gives the RT and MT calculated from figure 24 vs. the mole fractions of phosphatidyl choline and lysolecithin.

With a view to understanding whether the addition of lysolecithin to pure phosphatidyl choline gel has interaction in terms of the phase transition or not, a curve was constructed for X_{PC} = .51 from a weighted average of the transition curves of pure phosphatidyl choline and lysolecithin gels in figures 18 A and B. The result is given in figure 26. This was compared with the experimental curve for X_{PC} = .51 given in figure 24 IV. The constructed curve is broader than the experimental curve. The RT values for the experimental and constructed curves are 46-55° and 34-51° respectively. The middle transition temperature for the constructed curve is found to be 9° lower than that for the experimental curve.

It is interesting to note that the MT of curve I (X_{PC} = .96 and X_{LL} = .04) occurs at about 2° higher than that of pure phosphatidyl choline gel. As the X_{PC} is reduced to .63 two distinct transitions occur. It is also interesting to note that an almost equimolar mixture (curve IV X_{PC} = .51 and X_{LL} = .49) of PC and LL shows only one transition at 51° well above that of pure PC-gel and LL-gel. Figure 27 shows a phase diagram using the MT values of the mixed gels vs. X_{PC}. The dotted line in the figure indicates just an extrapolation as experiments with gels containing X_{PC} less than .51 were not performed.

Table V

Mole fractions of phosphatidyl choline and lysolecithin in mixed neutral lipid gels and their transition temperatures.

	Mole fraction PC	RT °C	MT °C	Mole fraction LL
	1.00	41.5-50	45.5	0.00
I	.96	45-49	47	.04
II	.83	40-50	45	.17
III	.63	27-34.5	31.5	.37
		47-54	50	
IV	.51	46-55	51	.49
	0.00	26-49	36	1.00

Figure 26

Theoretical curve constructed for PC-LL-water gel with X_{PC}=.51 and X_{LL}=.49 by using transition curves of pure PC gel and LL gel in figures 18 A and 18 B.

RT °C	MT °C
34-51	42

Sample with X_{PC} = .51

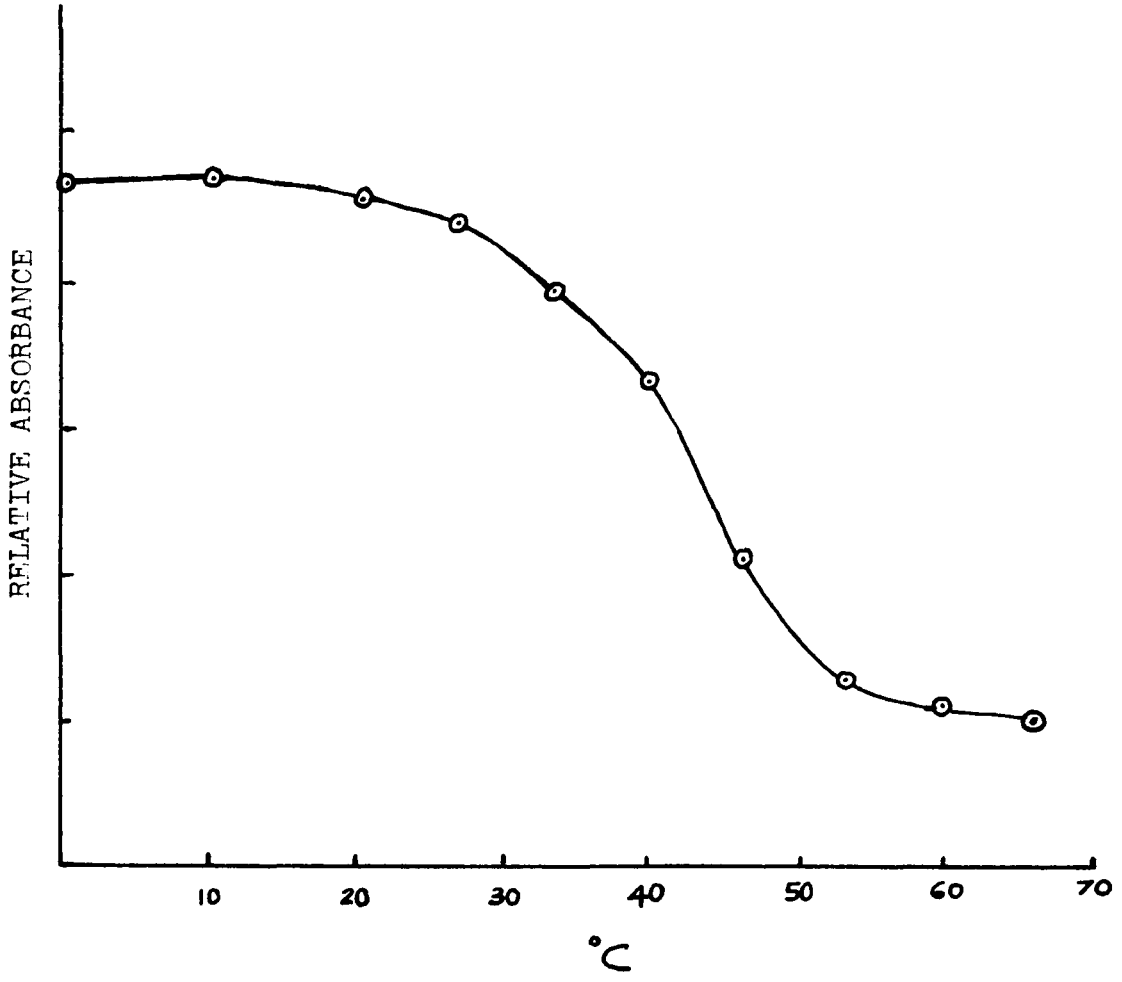
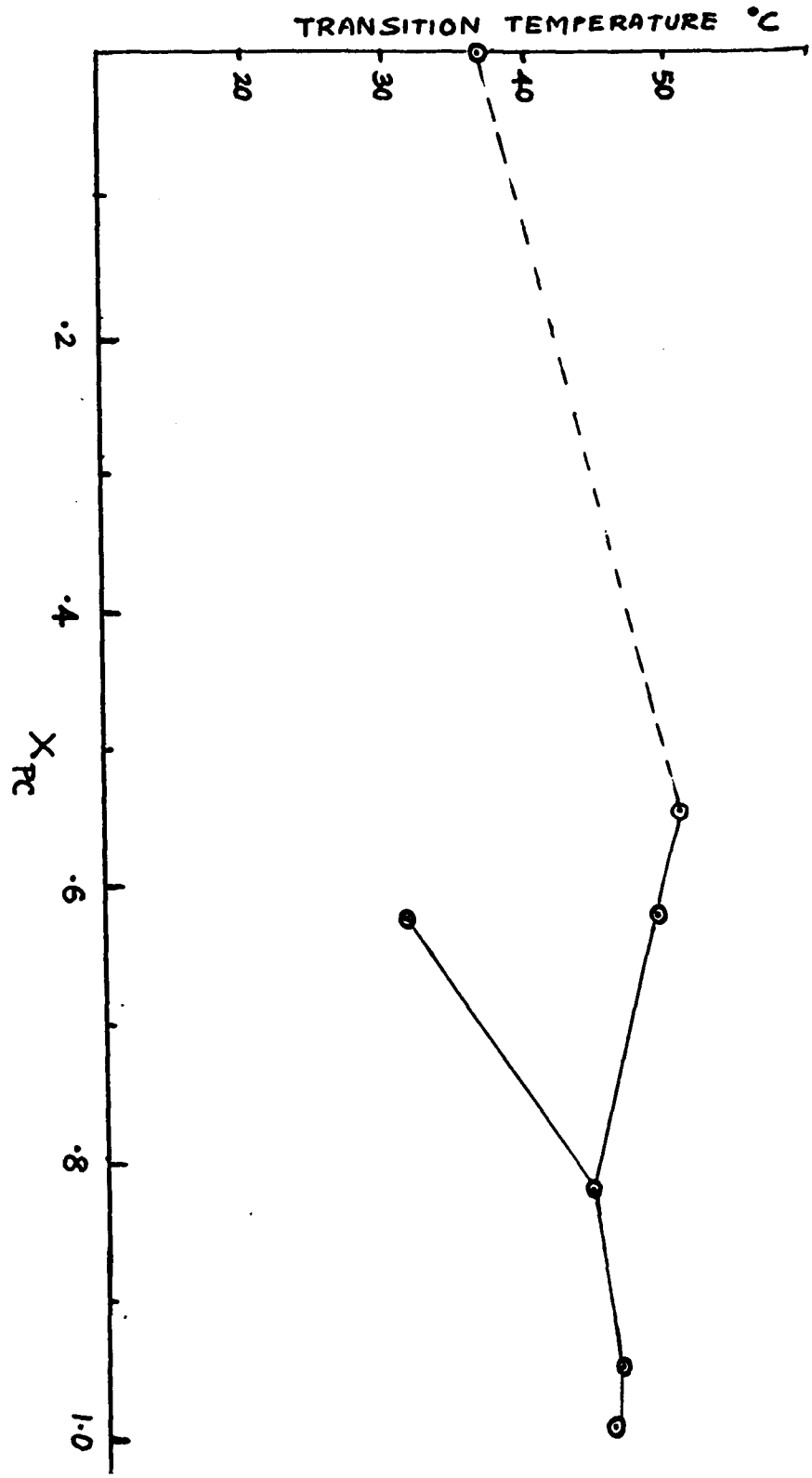


Figure 27

Data of Table V, got from figure 24, plotted as a phase diagram for PC-LL-water (mixture of neutral lipid-water system).


TRANSITION TEMPERATURE °C
20
30
40
50
·2
·4
·6
·8
1·0
X_{PC}

III Influence of anionic phospholipid on neutral lipid-water gels (Ternary Compounds) by infrared and Raman spectroscopy

The important phospholipids present in membranes are 1) lecithins or phosphatidyl cholines, 2) phosphatidyl ethanolamines, 3) phosphatidyl serines and inositols and 4) spingomyelins. Of these, phosphatidyl serines and inositols are anionic phospholipids. The general chemical formula of phosphatidyl serine is shown in figure 28 A.

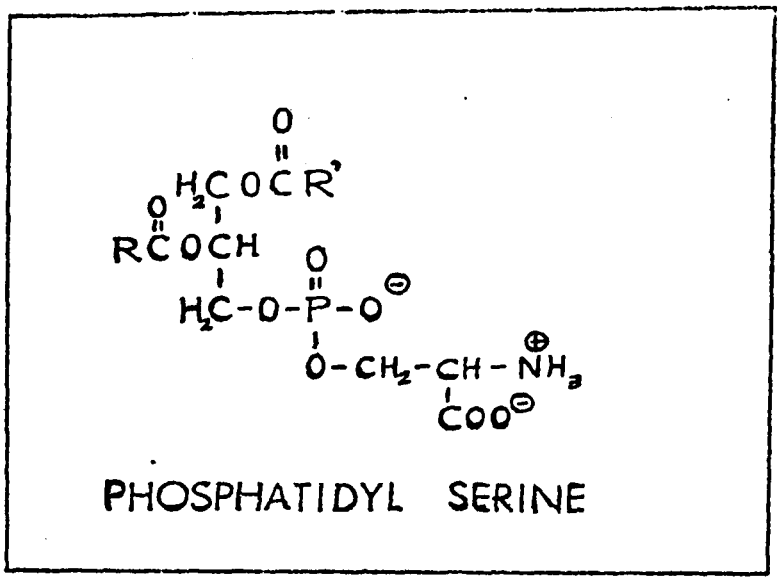
The potential importance of the presence of anionic phospholipids in membranes has been recognized for sometime[61]. It is found that in mitochondria membranes phosphatidyl serines constitute about 0.5% of the total phospholipid. Such membranes behave as cation exchangers for calcium, so they may provide negatively charged sites for the cation exchange. This also suggests an important role for the phosphatidyl serines in active transport, despite the fact that they are present in minute amount in relation to the total phospholipid content of some of the membranes.

The influence of the anionic phospholipid on the thermal phase transition of the mixture of phospholipid-water (20% water) was investigated. Infrared spectra (600-4000 cm^{-1}) of such mixtures with varying ratios of phosphatidyl serine (PS) and phosphatidyl choline (PC) are taken as a function of temperature.

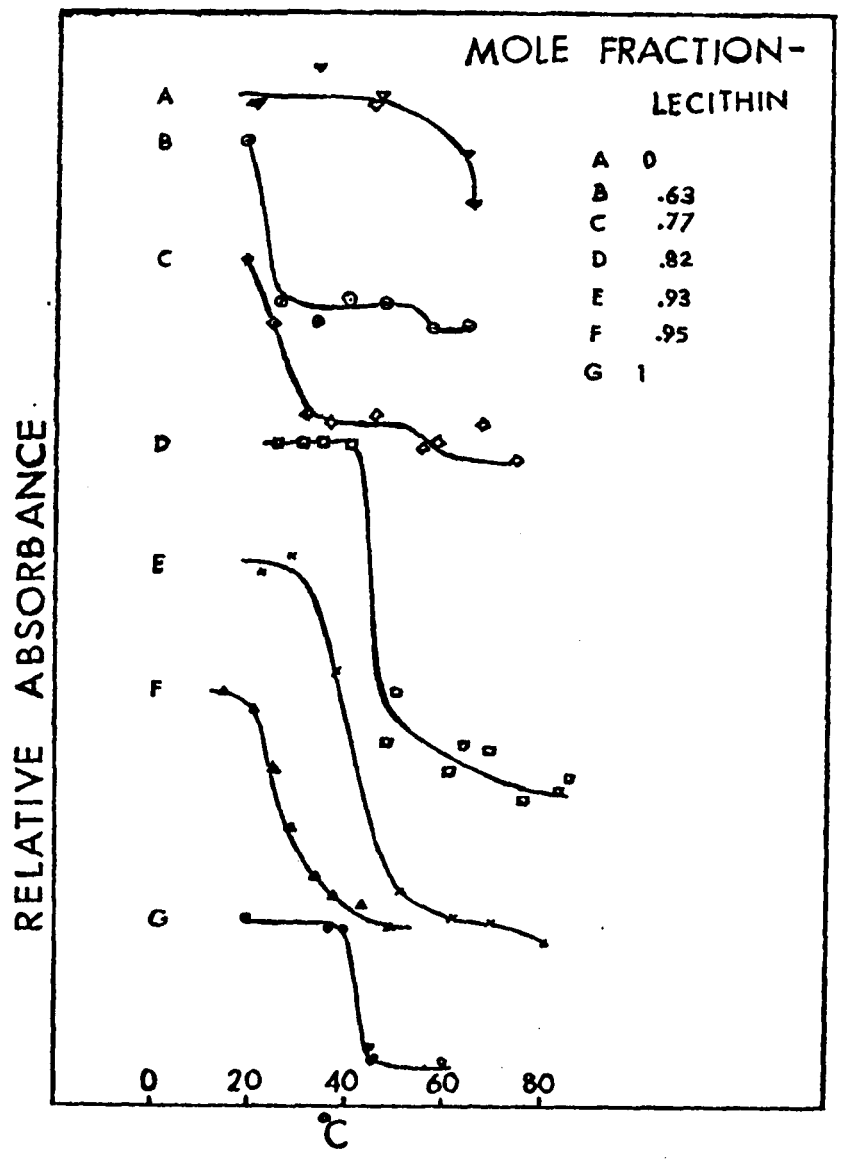
Figure 28

A General chemical structure of phosphatidyl serine

B Relative absorbance of infrared band at 1470 cm^{-1} vs. temperature of PC-PS-H_2O gels containing varying mole fractions of PC.



PHOSPHATIDYL SERINE



Spectra were obtained from -20 to 80° with proper care taken not to destroy the gel.

Figure 28 B shows the plot of the peak intensity of the 1470 cm^{-1} band (methylene deformation vibration), relative to that of another band in the spectrum, as a function of temperature in the ternary mixtures phosphatidyl serine-lecithin-water. At low mole fraction (up to 0.05) the phosphatidyl serine depresses the transition temperature (OT) from the lecithin-water value of 41°. Above this eutectic point, the increase in phosphatidyl serine mole fraction causes a continuing rise in the transition temperature. At 0.23 and 0.37 mole fraction of phosphatidyl serine one can observe two transitions, one at low temperature (approximately 20°) and another at high temperature (approximately 50°).

Table VI gives the mole fraction of phosphatidyl serine with respect to lecithin in the ternary mixture along with the respective phase-transition temperature in those ternary mixtures. From Table VI a plot of transition temperature vs. mole fraction of lecithin was constructed and it is given in figure 29. The phase diagram indicates the presence of three phases at the point when the phosphatidyl serine mole fraction is about .23.

A thermogram (DTA) of pure phosphatidyl choline-water mixture was run from 10-70°. From this curve the ΔH_f was

Table VI

Mole fraction of phosphatidyl serine with respect to lecithin in ternary mixtures and their respective phase-transition temperature. Amount of water in all samples are kept constant around 20%.

	Mole fraction PS	RT °C	MT °C	Mole fraction PC
A	1.00		61	0.00
B	.37	18-26	21.5	0.63
		54-57.5	55	
C	.23	19-32	26	0.77
		51.5-60	56	
D	.18	42-46	44.5	0.82
E	.07	31-50	40.5	0.93
F	.05	20.5-31	26.5	0.95
G	.00	40-46	43	1.00

RT : Range of transition temperature

MT : Mid-transition temperature

Table VI

Mole fraction of phosphatidyl serine with respect to lecithin in ternary mixtures and their respective phase-transition temperature. Amount of water in all samples are kept constant around 20%.

	Mole fraction PS	RT °C	MT °C	Mole fraction PC
A	1.00		61	0.00
B	.37	18-26	21.5	0.63
		54-57.5	55	
C	.23	19-32	26	0.77
		51.5-60	56	
D	.18	42-46	44.5	0.82
E	.07	31-50	40.5	0.93
F	.05	20.5-31	26.5	0.95
G	.00	40-46	43	1.00

RT : Range of transition temperature

MT : Mid-transition temperature

Figure 29

Data of figure 28 B (Table VI) plotted as a phase diagram for the PC-PS-H_2O system.

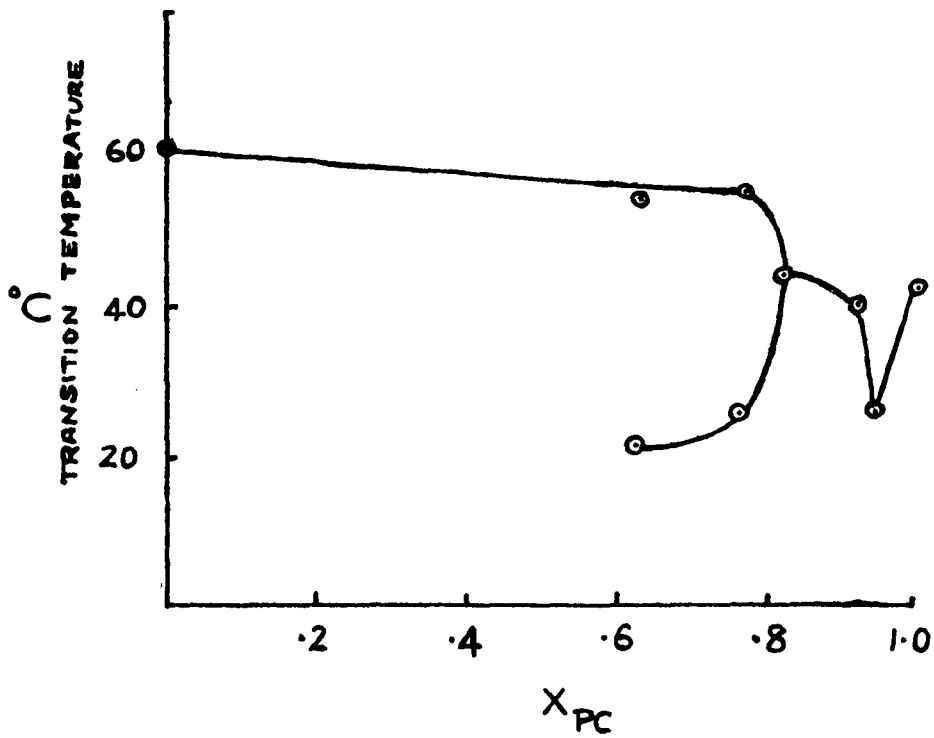

TRANSITION TEMPERATURE
°C
60
40
20
·2
·4
·6
·8
1·0
X PC

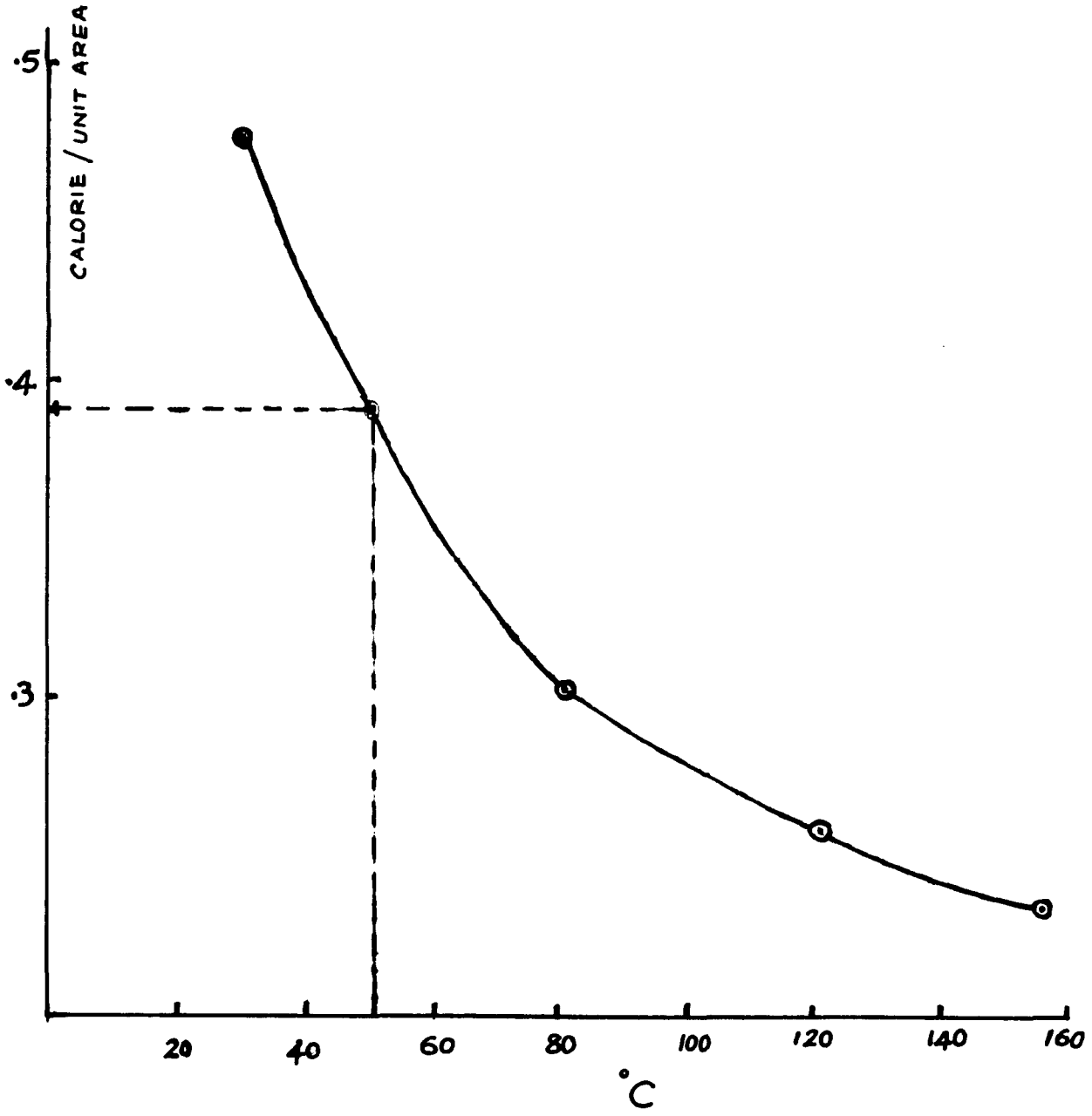
Table VII

Data from thermograms of some standards and lipid gel

Compound	M.Pt C	Mass (g)	H_f cal/g	cal.	area	cal./area
Benzoic acid	121.8	.00046	33.0	.015594	60	.00026
Indium	156.3	.00121	6.8	.008228	35	.0002351
Naphthalene	80.2	.00109	35.06	.0382154	126	.0003033
Gallium	29.0	.00312	19.1	.059592	125	.0004767
Unknown L+H_2O	53.0	.00292L .00068 H_2O	16.38	.04914	126	.00039 (obtained from figure 30)

Figure 30

Data from differential thermal analysis (Table VII) plotted as calories per unit area vs. transition temperature.


CALORIE / UNIT AREA
·5
·4
·3
20
40
60
80
100
120
140
160
°C

found to be 16.38 cal/g (12.02 kcal/mole). Figure 30 shows the graph constructed from Table VII in order to calculate the ΔH_f for phosphatidyl choline-water mixture. Assuming ideal solution behavior, a freezing point depression calculation was carried out for phosphatidyl choline-phosphatidyl serine-water mixture in which phosphatidyl serine mole fraction was .05. The depression in MT by that calculation was predicted to be $\sim 1^{\circ}$. However, the infrared measurement reveals that the presence of .05 mole fraction of phosphatidyl serine in the lecithin-water gel depresses the transition temperature (MT) by 16°. Comparison of RTs for lipid gel sample (PC gel) and lipid gel sample containing .05 mole fraction of phosphatidyl serine (PS-PC gel) reveals that the transition of PS-PC gel is as sharp as that of PC-gel. This indicates the presence of unique phase in PS-PC gel containing .05 mole fraction of PS.

IV Cytochrome C complex with lipid-water gels

Proteins present in membranes have been characterized as extrinsic and intrinsic[62]. The intrinsic proteins are distributed across the thickness of the membrane whereas the extrinsic proteins are in the plane of the membranes. The intrinsic proteins are considered to be globular and bimodal. They may be either monomeric or multimeric and may exist in either an ordered or disordered arrangement.

The interaction of these proteins with lipids has been studied and lipo-protein complexes isolated, by various workers[63, 64]. Of these proteins, cytochrome C was also found to form lipo-protein complexes either in the reduced or oxidized form. Soluble and insoluble lipo-protein complexes of this protein have been isolated[65-70].

Cytochrome C is a small hemoprotein of molecular weight 12,400. The structure and history of this ancient protein have been reviewed by Dickerson[71]. This molecule exists in a variety of forms and, in one form or other, is responsible for the oxidation of food molecules in all organisms from yeast to man. This protein contains around 104 amino acid units. These amino acid units are linked in a continuous chain that grips and surrounds a heme group.

A more detailed structural analysis[72] of cytochrome C from various species (about 29) have been done by X-ray

methods. The differences between heart cytochrome C and the cytochrome C derived from other species is attributed to differences in the amino acid sequences and the number of amino acid units, but the heme group remains the same. The horse heart cytochrome C has a polypeptide chain of 104 amino acids. This chain is wrapped around the heme group in two halves. The residues 1 to 47 are located to the right and 48 to 91 are located to the left of the heme. Residues 92 to 104 form an helical strap rising over the top rear of the molecule and back across the right side again.

Cytochrome C is an electron-carrying protein found in mitochondria of all aerobic organisms. The main function of this protein is in the terminal oxidation chain achieving the breakdown of foods to CO_2 and H_2O and storing the liberated energy in molecules of ATP. As said earlier this is an iron porphyrin protein, made up of a heme group and a polypeptide chain. The iron atom alternates between the +2 and +3 oxidation states as the molecule interacts in succession with cytochrome oxidase and cytochrome reductase, each a large multi-molecular enzyme catalyzing the reaction by an electron-transfer mechanism.

Many physical techniques, such as ESR spectroscopy[72], optical rotatory dispersion[73], X-ray scattering studies[74] and spectrophotometric studies[75], have been employed to

study the lipid-cytochrome C complexes.

In the ESR study[72], complexes of lipid with spin labeled protein indicated that an ionic combination of phospholipids and proteins was present. The freedom of motion of the spin-label molecules depended on the hydrophobic property of phospholipid chains. ESR study of isooctane soluble complexes of lipid-protein also suggested the complexation of an individual protein molecule with several phospholipids.

A plot of specific rotation vs. wavelength in the 300 nm - 600 nm region indicated [73] that the cytochrome C in water and the cytochrome C - phospholipid complex in isooctane solvent gave identical plots, thus indicating that formation of the complex with phospholipid does not alter the rotatory properties of the cytochrome C.

Small angle X-ray scattering studies[74] of cytochrome C-phospholipid complexes in isooctane suggested not only the presence of 20:1 and 30:1 lipid-protein monomeric units, but also complexes due to molecular aggregation (particle weight $>10^6$).

Figures 31 A and 31 B give the spectra of cytochrome C in oxidized as well as reduced form in aqueous solution. The reduced cytochrome C was prepared by the addition of a few crystals of sodium dithionite. It was found that as little as two-fold excess of sodium dithionite (mole

Figure 31

A Spectra of oxidized cytochrome C in aqueous solution in the region 300-600 nm.

B Spectra of reduced cytochrome C in aqueous solution in the region 300-600 nm.

ABSORBANCE
300
375
450
525
600
mμ
A
B

basis) was sufficient. The following ionic reaction indicates the stoichiometry for the reduction of cytochrome C with dithionite:

$$S_2O_4^{-2} + 2Fe^{+3} + 2H_2O \longrightarrow 2SO_3^{-2} + 2Fe^{+2} + 4H^+$$

According to the above reaction, 2 moles of cytochrome C can be completely reduced by the addition of 1 mole of sodium dithionite. Solutions of dithionite ion $S_2O_4^{-2}$ are not very stable and decompose by disproportionation.

$$2S_2O_4^{-2} + H_2O = S_2O_3^{-2} + 2HSO_3^-$$

Decomposition is rapid in acid solution producing elemental sulphur. It is thus necessary to add dithionite in crystal form and also in calculated amounts, thus avoiding a large excess. In all experiments, where a reduced cytochrome C was required, a two-fold excess of dithionite was used just to make sure of complete reaction.

The spectrum of oxidized cytochrome C has a strong absorption band at 410 nm whereas that of reduced cytochrome C has an α band at 550 nm and a β band at 520 nm in addition to a strong band at 410 nm. The band at 410 nm was used for quantitative estimation of cytochrome C in solution. The spectra were taken at room temperature using a Cary 14 spectrophotometer. The band at 410 nm is called the 'soret' band which arises due to pure electronic excitation, whereas the α band is due to electronic-vibronic excitation and the β band is due to vibronic excitation.

Preparation of insoluble complexes with reduced and oxidized cytochrome C and phospholipid

Das et al[68] suggested the formation of insoluble complexes when the amount of phospholipid added exceeds the amount required for complete extraction of cytochrome C into the isooctane layer. At this point they found a decrease in the amount of cytochrome C in the isooctane. Thus the presence of an excess of complex forming phospholipid leads to the formation of a new type of isooctane insoluble cytochrome C complex.

An insoluble complex of oxidized and reduced cytochrome C with phospholipid was prepared as follows: A calculated amount of lipid (lipid: L-2,3 dipalmitoyl glyceryl-N,N dimethyl phosphorylamine) was weighed and dispersed in a known volume (5 ml) of distilled water. For dispersion of lipid a sonicator with a microtip was used. For the first 5 minutes the sonication was done at 75% power and then for additional 5 minutes at reduced power. This avoided the excessive generation of heat due to sonication. A calculated amount of cytochrome C was dissolved in a known volume of distilled water and stored in sealed tubes with sides covered by black paper. Both the lipid dispersion and the cytochrome C solutions were stored in the refrigerator.

Two well cleaned centrifuge tubes, which can withstand

as high as 3 X 10^4 revolutions per minute were taken. To each one of them 1 ml of cytochrome C solution was pipetted out. One ml of cytochrome C solution contains approximately 109 μmole cytochrome C. To one of the centrifuge tubes marked 'R' one or two crystals of sodium dithionite was added and mixed by gentle swirling. One could observe the immediate color change from pink to pale red in the tube marked 'R'. Then to each one of these tubes (O and R marked tubes) 2 ml of lipid dispersion was added. Two ml of lipid dispersion contain close to 200 μmole of lipid. As soon as the lipid dispersion was added an insoluble precipitate was formed. One tube marked 'O' had a pink colored precipitate and the other tube, marked 'R' had a pale red colored precipitate. The tubes were sealed with rubber stoppers, mounted in a shaker and shaken for 10 to 15 minutes at room temperature. After the shaking was completed, the mixtures were centrifuged for 1 hour at a temperature of 20° and at a speed of 10^4 revolutions per second. After the centrifugation was complete a known volume of the supernatant liquid was pipetted out in two 10 ml volumetric flasks, made up to volume with water, and the visible spectra were taken between 300-600 nm using the Cary 14 spectrometer. The spectra were taken against distilled water. A known volume of original cytochrome C solution was also pipetted out in 10 ml volumetric flask, made up to the volume with water, and its spectrum also taken against distilled water between 300-600 nm.

Figure 32

Change in absorbance (relative to another band in the spectrum) of infrared band at 1470 cm^{-1}, as a function of temperature, for :

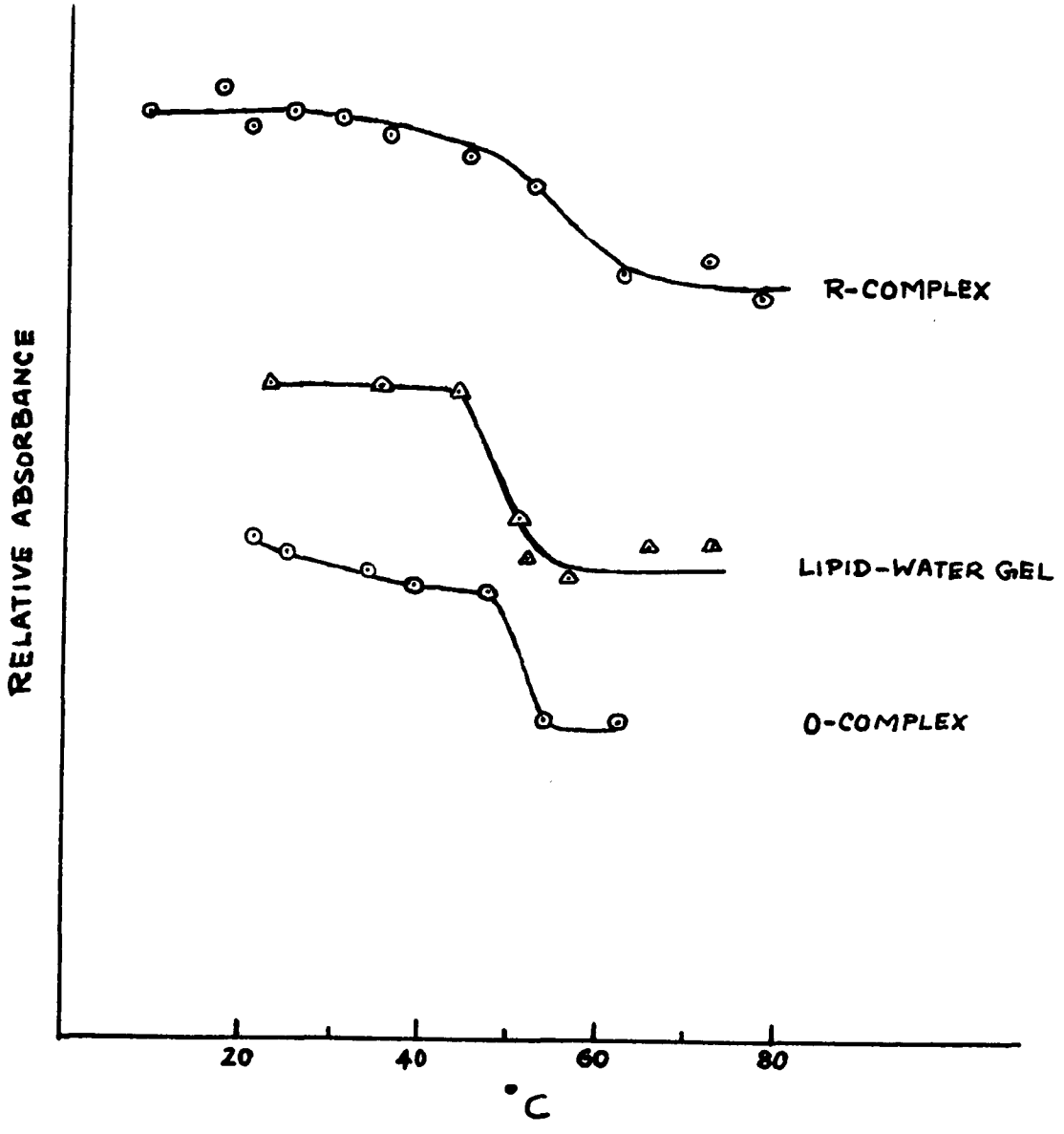
A Reduced cytochrome C-lipid-water complex.

B Lipid-water gel

Lipid used : L 2,3 dipalmitoyl glyceryl-NN dimethyl ethanolamine.

C Oxidized cytochrome C-lipid-water complex.

Sample	RT °C	MT °C
R-Complex	42.5-63.5	53.5
Lipid gel	44-53	48
O-Complex	48-54	51



By comparison of the peak height at 410 nm, 22.5% of reduced cytochrome C was found to be present in the precipitate in the tube marked 'R' and 43.2% of oxidized cytochrome C was found to be present in the precipitate in the tube marked 'O'. It was found by calculation that the ratio of mole of lipid:mole of reduced cytochrome C was 80:1 and the ratio of mole of lipid:mole of oxidized cytochrome C was 44:1.

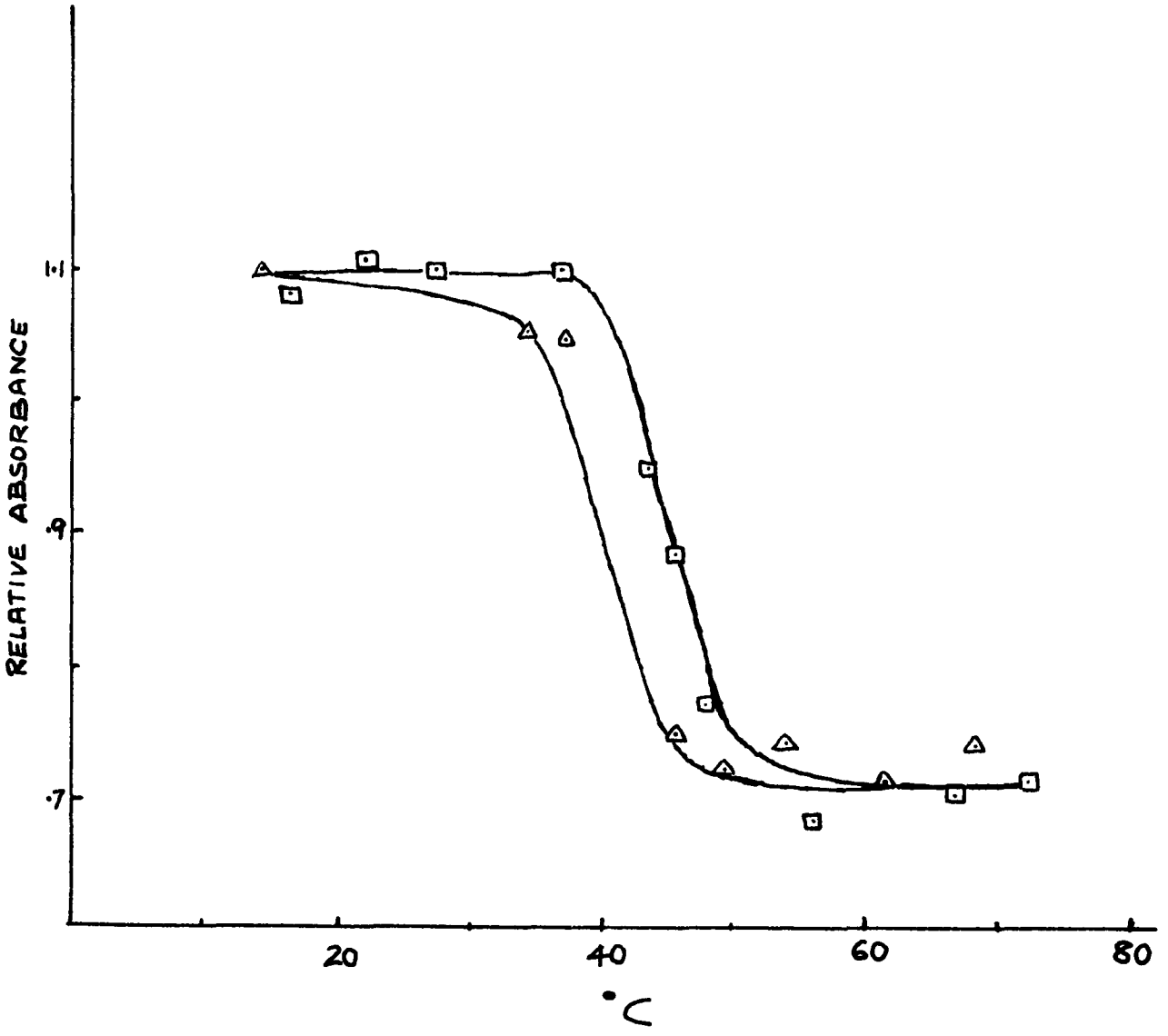
Infrared spectra of the lipid gel, oxidized cytochrome C-lipid complex and the reduced cytochrome C-lipid complex were taken with varying temperature. From the spectra the ratio of relative absorbance against the temperature were plotted. This is given in figure 32. From these curves the mid transition temperature for R-complex, lipid gel, and the O-complex, were estimated to be 53.5 , 48 and 51 . It is interesting to note that both lipid gel and the O-complex have relatively sharp transition when compared to the R-complex which is very broad. The results (RT & MT) are shown along the side of the figure 32.

V Hysteresis effect observed in lipid-water gel

A lipid gel (lipid: synthetic lecithin) prepared with 20% water was chosen for this study. The lipid gel was cooled to 14°. The infrared spectra of the sample were taken at various temperatures after 30 minutes of equilibration at each temperature. When the sample temperature reached 72°, the sample was then cooled. The infrared spectra were taken at various temperatures until the sample temperature again reached 14°. A plot of the relative absorbance vs. temperature gave curve resembling hysteresis curve. This plot is shown in figure 32D. The heating curve has an ET-OT of 11.5° and the cooling curve has an ET-OT of 12°. The MT of the heating curve is 45° while the MT of the cooling curve is 40°.

Figure 32D

Relative absorbance vs. Temperature plot of lipid gel.
(Hysteresis plot)
Heating curve is on the right side.
Cooling curve is on the left side.


RELATIVE ABSORBANCE
1
.9
.7
20
40
60
80
°C

VI Differential thermal analysis and other techniques used to study such model membranes

The thermotropic mesomorphism exhibited by phospholipids was discovered by differential thermal analysis as early as 1965. A complete review of differential thermal analysis of lipids, proteins and biological membranes have been given elsewhere[40]. This technique has been used elaborately to determine the endothermic phase transition of the lipid gels, model membranes and biological membranes as well[37-39]. It was found that these thermal transitions are reversible and involve chain melting and depend upon the cooperative assiciation between the lipid hydrocarbon chains.

Many of these works formed a basis for the study of the lipid systems by spectroscopic techniques[50-53]. The thermal studies have revealed the existence of more than one thermal transition when pure phospholipid or lipid gel is heated.

Figure 33 shows some thermograms of lipid gel samples. Many thermograms of phosphatidyl choline-phosphatidyl serine were also taken. Figure 34 shows some of them. LL-gel undergoes a rather broad transition, whereas PC-gel undergoes a sharp transition. All these thermograms were taken against glass beads (standard). Getting a thermogram of neutral lipid-PS gel was difficult. Because PS was available in benzene solution, the evaporation of the solvent

Figure 33

A Thermogram of PC gel

B Thermogram of L 2,3 dihexadecyl α glyceryl NN dimethyl phosphoryl cholamine gel

C Thermogram of LL gel

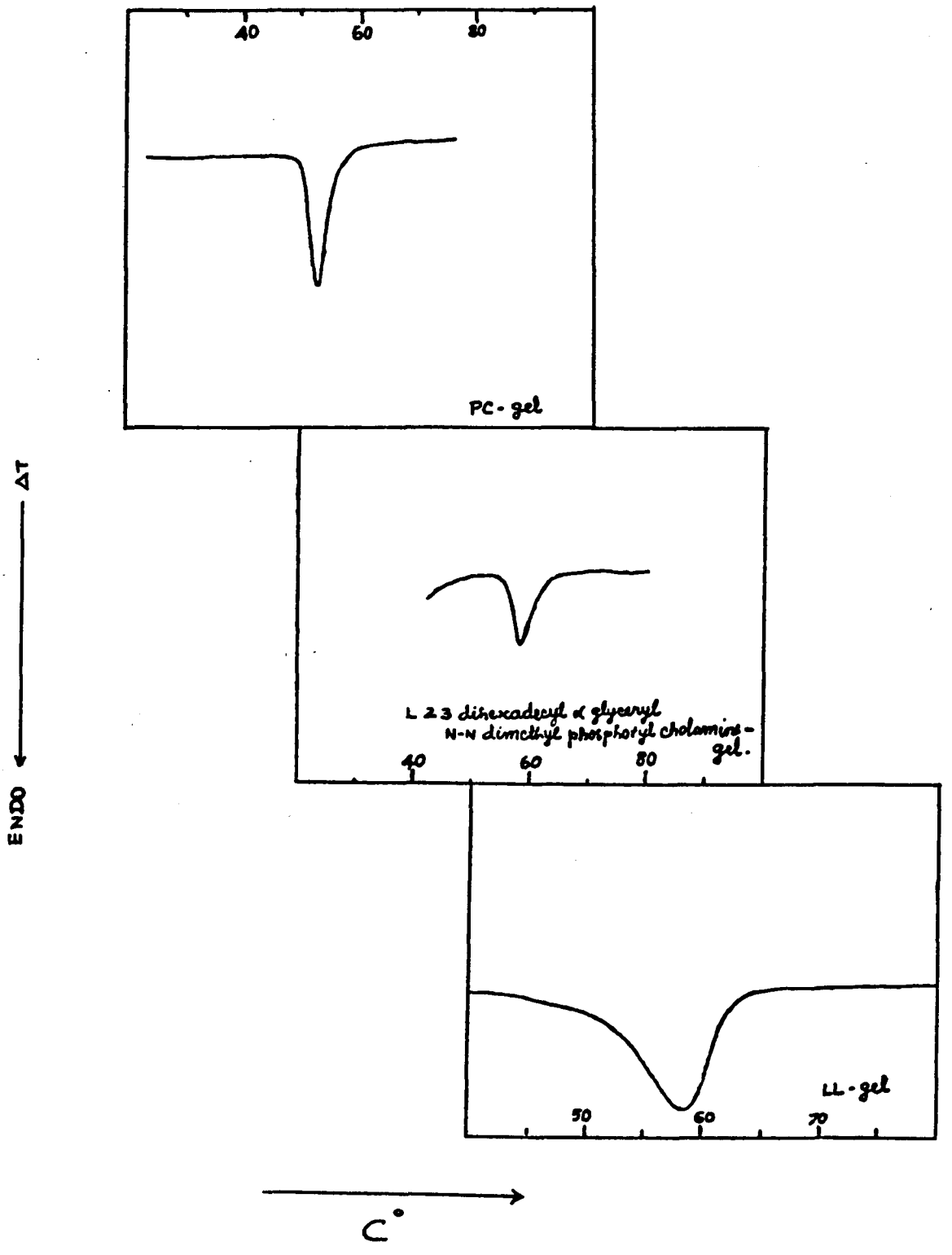

40
60
80
PC - gel
L 2 3 dihexadecyl α glycaryl
N-N dimethyl phosphoryl cholamine -
gel.
40
60
80
50
60
70
LL - gel
ENDO
ΔT
C°

Figure 34

Some thermograms of PS-PC-water system

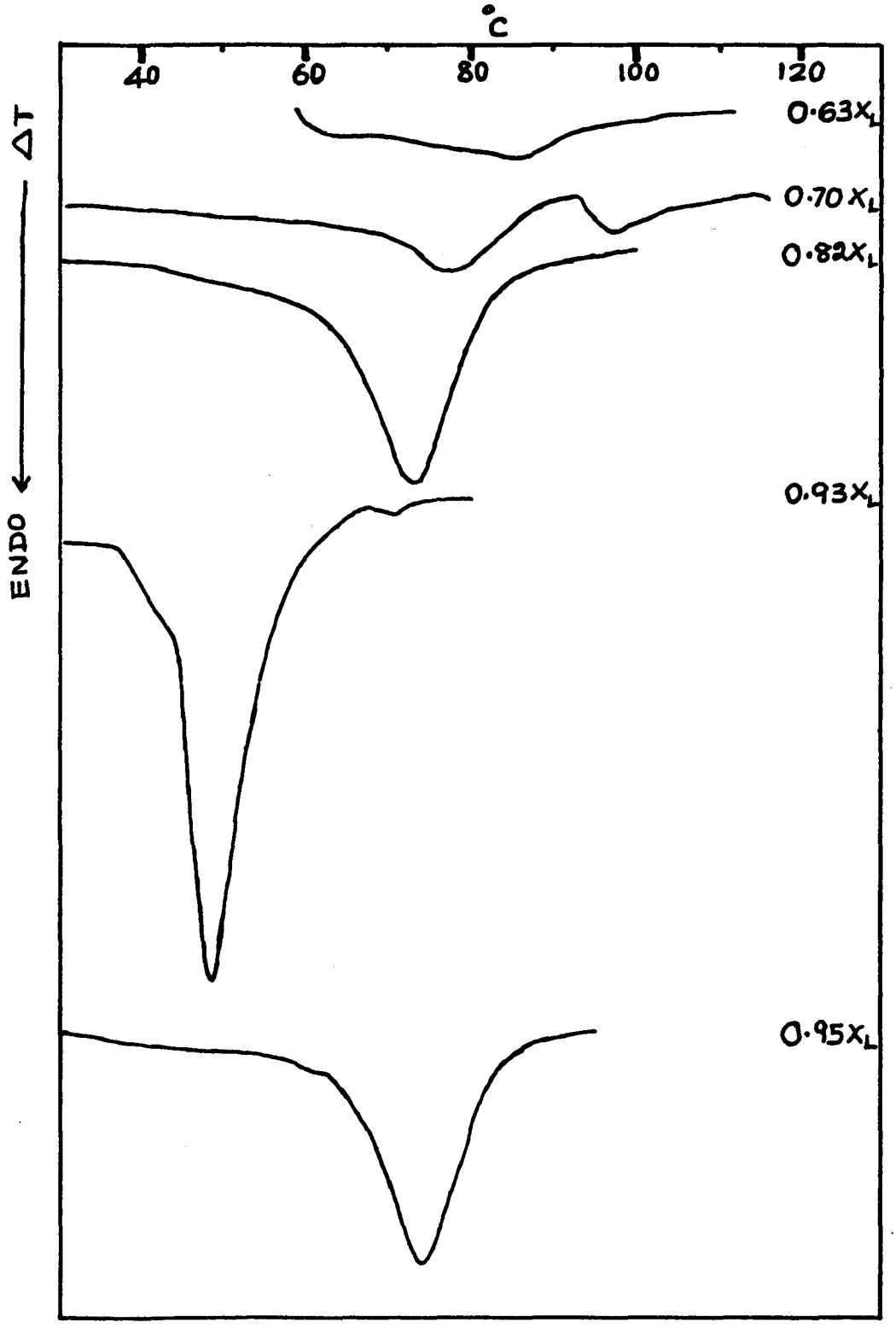

°C
40
60
80
100
120
ΔT
ENDO
0·63X_L
0·70X_L
0·82X_L
0·93X_L
0·95X_L

Figure 35

Transmitted light vs. temperature of LL gel

a Sample taken in a capillary tube.

b Sample taken in capillary tube with a thin micro glass rod inserted inside.

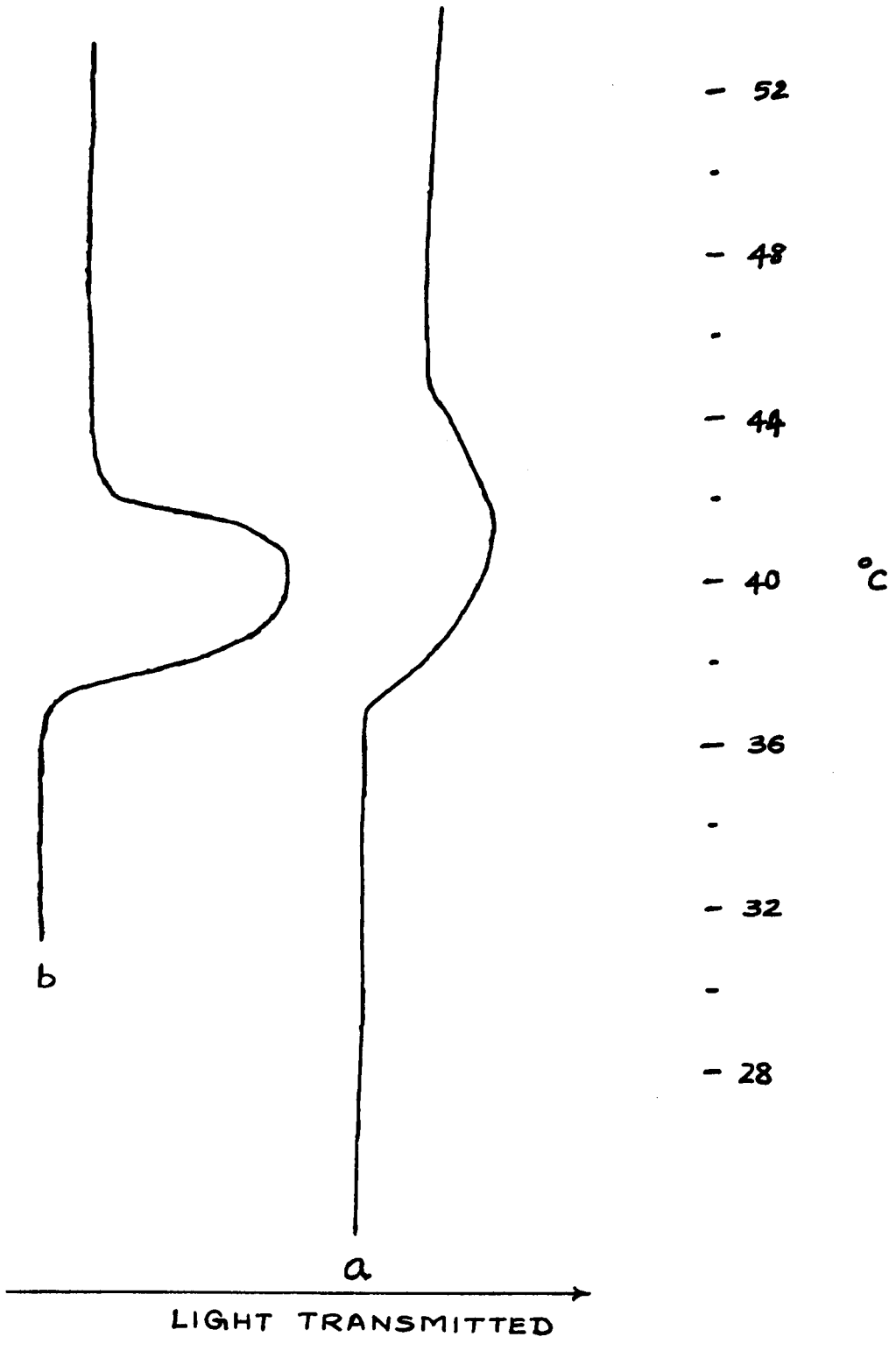

52
48
44
40
36
32
28
°C
b
a
LIGHT TRANSMITTED

figure 36

Scattered light of a lipid gel sample

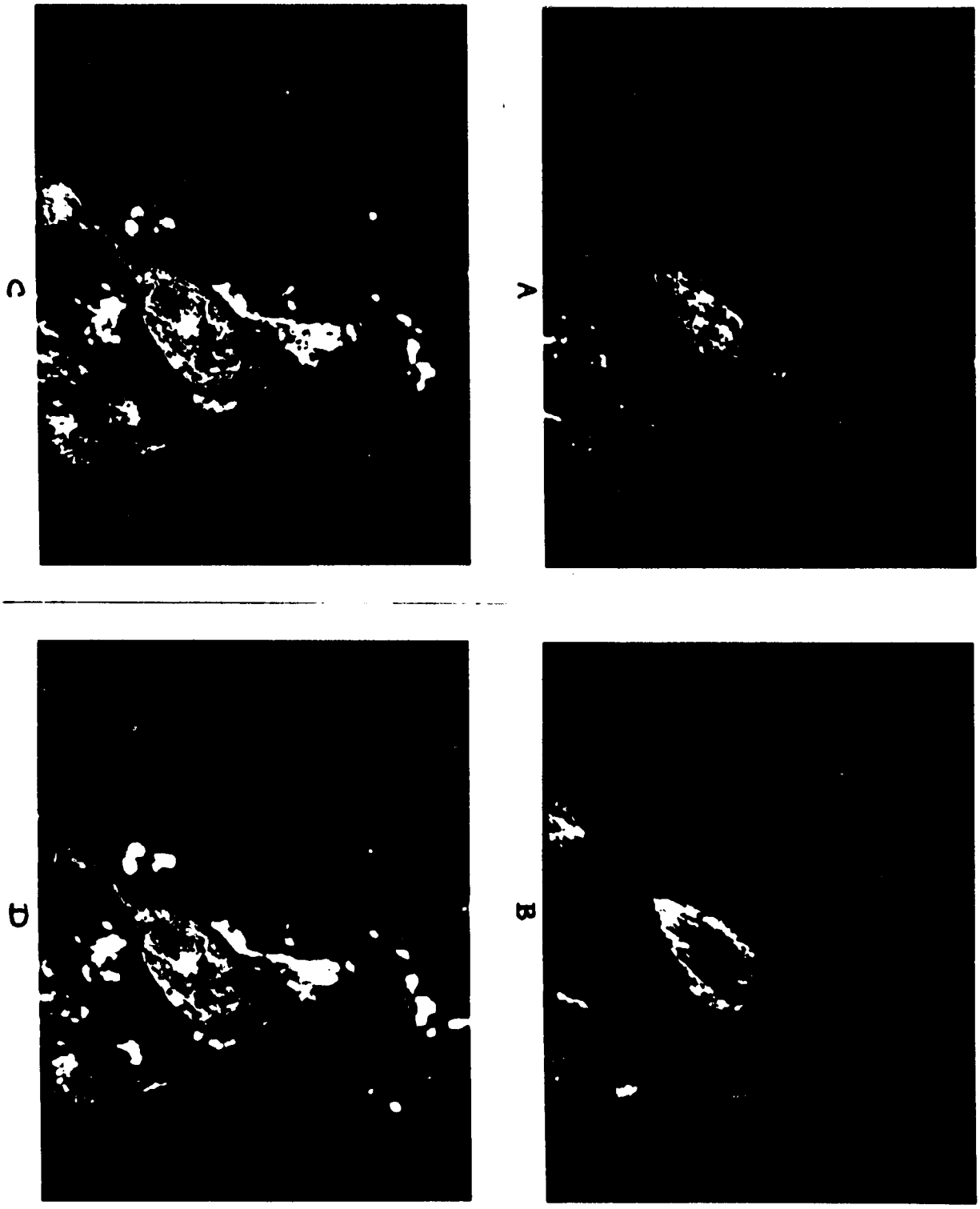
A at 30.3°

B at 51.7°

C at 61.4°

D at 75.5°

Lipid used : lysolecithin


A
B
C
D

was absolutely necessary to get a good thermogram.

Some optical studies of these model membranes were also done using the modified Mettler FP2 melting point apparatus shown in figure 10. The lysolecithin gel was placed in a 0.5 mm diameter capillary tube. The sample was heated slowly at a rate of 2°/min. and the transmitted light was amplified and fed to a recorder. Figure 35 shows the graph relating to the amount of transmitted light as the temperature of the sample was increased. Once the sample temperature had reached about 70°, it was cooled and the experiment was repeated. The same plot was obtained as shown in figure 35 A. Figure 35 B shows the plot when the experiment was repeated by inserting a thin uniform glass rod into the capillary tube containing the sample.

Instead of containing the lipid gel in a capillary tube and observing the transmitted light, the sample was placed below a light source and the reflected light was observed by a microscope. Photos were taken of the sample at different temperatures by Polariod camera. The LL-gel sample was used for this experiment. The pictures 1, 2, 3 and 4 shown in figure 36 give the photos of the sample at 30.3°, 51.7°, 61.4° and 75.5° respectively.

DISCUSSION

I The role of lipid hydrocarbon chains in the mesomorphic phase transition

An important difference between many membrane models suggested for the arrangement of proteins and lipids in biomembranes is the degree to which the lipid bilayers, with ordered fatty acid chains, play a role in the structure. It is a speculation that increased knowledge about the mobility in these fatty acid chains would lead to an understanding of the significance of the lipids in determining membrane properties.

Results from infrared and Raman spectroscopy indicate that molecular vibrations can be a sensitive probe of order in the fatty acid chains of phospholipids. As indicated earlier, the number of bands observed, the relative intensity, and the band widths, all yield information on this subject.

Chapman[54] has shown that when a phospholipid in a KBr pellet is heated to 140°, the bands broaden and a number of bands disappear. This is similar to the change seen between figures 13 and 14. Thus, in the gel at room temperature the phospholipid appears to be in a liquid-like state with respect to the time scale of molecular vibrations. The broad bands seen in figure 14 are typical of systems in which a number of slightly different environments exist. This is undoubtedly the case for the lipid glycerol back-

bone and phosphoryl choline end, with its water sheath.

In evaluating the infrared and Raman spectra of these phospholipid systems as a whole, taken during the phase transition of these gels, it is important to look into the following three changes: intensities, frequencies and band widths. These all yield information on the role played by hydrocarbon chains in these systems.

The methylene deformation vibration at 1470 cm^{-1} (figure 37) remains as sharp in the gel as it was in the solid. This can be interpreted as indicative of a crystal-like environment for the methylene groups of the fatty acid chains. As discussed below, the Raman spectra yield more detailed information on this point.

As the temperature is raised, this environment changes, and the changes are reflected in a change in the intensity of the band at 1470 cm^{-1}. The decreasing intensity observed could mean that the overall dipole change for this transition is decreasing. One can speculate that this is due to a more random orientation of the chains. Alternatively, the less ordered chains may be shifting intensity into the wings of the absorption band away from the center. This would appear as a decrease in the peak intensity at 1470 cm^{-1}. It is this latter quantity which is plotted in figure 18.

One notes from figure 18 that the intensity change is

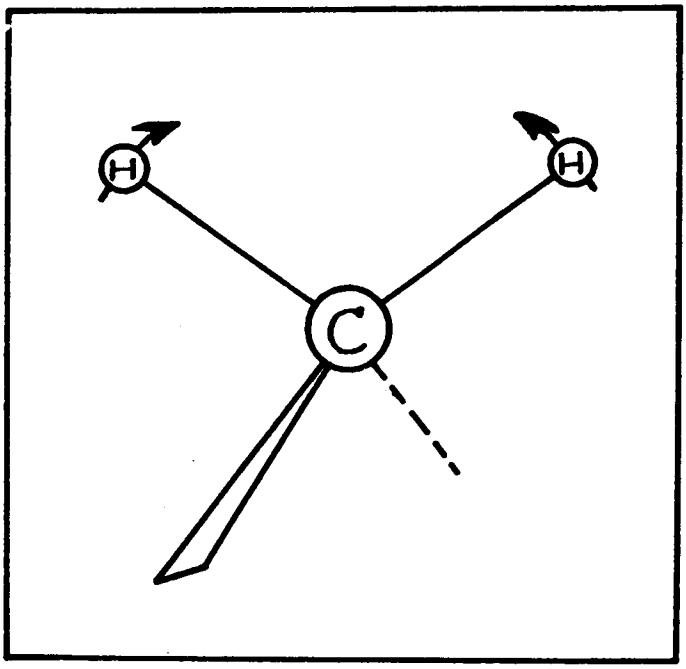
Figure 37

a. Methylene deformation vibration

b.

A Methylene symmetric vibration

B Methylene asymmetric vibration


H
H
C

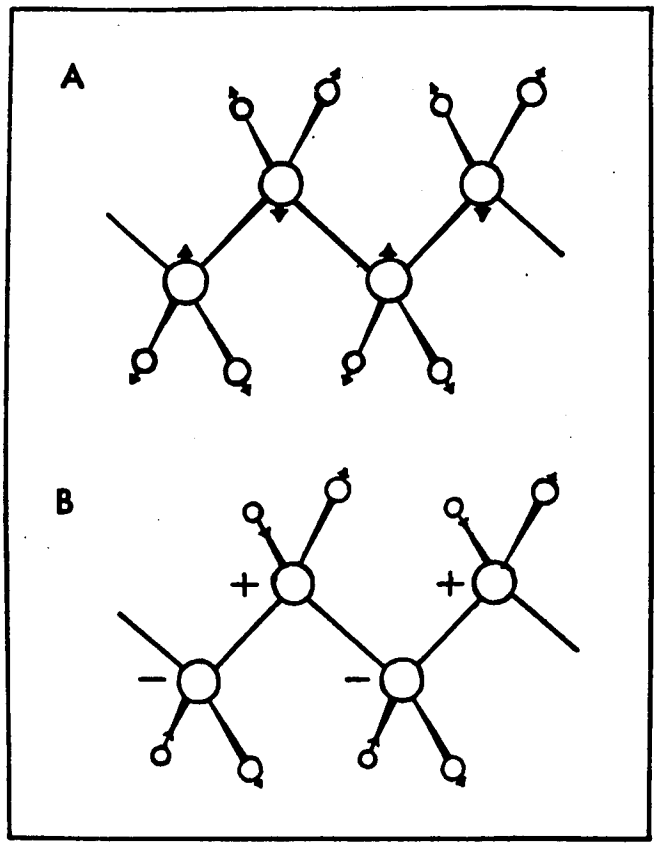

A
B
+
+
−
−

Table VIII

Transition temperatures for phospholipid-water systems[d].

Lipid	This work RT °C	MT °C	OT °C	Literature °C
Dipalmitoyl lecithin	43.5-47.5	45.5	43.5	41[c]
Lysolecithin	27-49	38	27	35[a]
Phosphatidyl ethanolamine	12-18[e]	15[e]	12[e],33	20[a], 25-35[b],55[a,b]
Lysophosphatidyl ethanolamine	43-53[e]	50[e]	9,43[e]	

a) From reference 56.

b) From reference 32.

c) From reference 40.

d) See experimental techniques for description of the phospholipids used in this study.

e) Transition temperatures refer to major transition of the system (refer to figure 18 a-d).

a sensitive method for detecting phase transitions in the phospholipid-water systems. It is difficult to make comparisons with transition temperatures from the literature, as these have not always been measured with pure phospholipids, or with the same fatty acid chains present. However, Table VIII summarizes the results from several key references and compares them with this work. As the transitions are not very sharp in some of these gels, the range of temperature and mid temperature at which the transition occurs are all shown in that table. As expected, some differences are found. This is usually the case for measurements made by widely differing techniques.

It is important to point out that the infrared method shown here is nondestructive to the system, is sensitive to small changes (e.g., the several PEA transitions), requires little sample, and involves no chemical perturbation. As with many spectroscopic techniques, it measures the transition as it affects one protion of the system, the CH_2 groups.

The transition temperatures measured here correlate well with the electron spin resonance spectra of Hubbell and McConnell[45]. This can be seen by comparing the results for PC-water gel from infrared spectra (figure 18 A) with those in reference 45 (shown in figure 38). Not only does the transition temperature agree closely, but the shape of the curve is similar as well. It is important to point

Figure 38

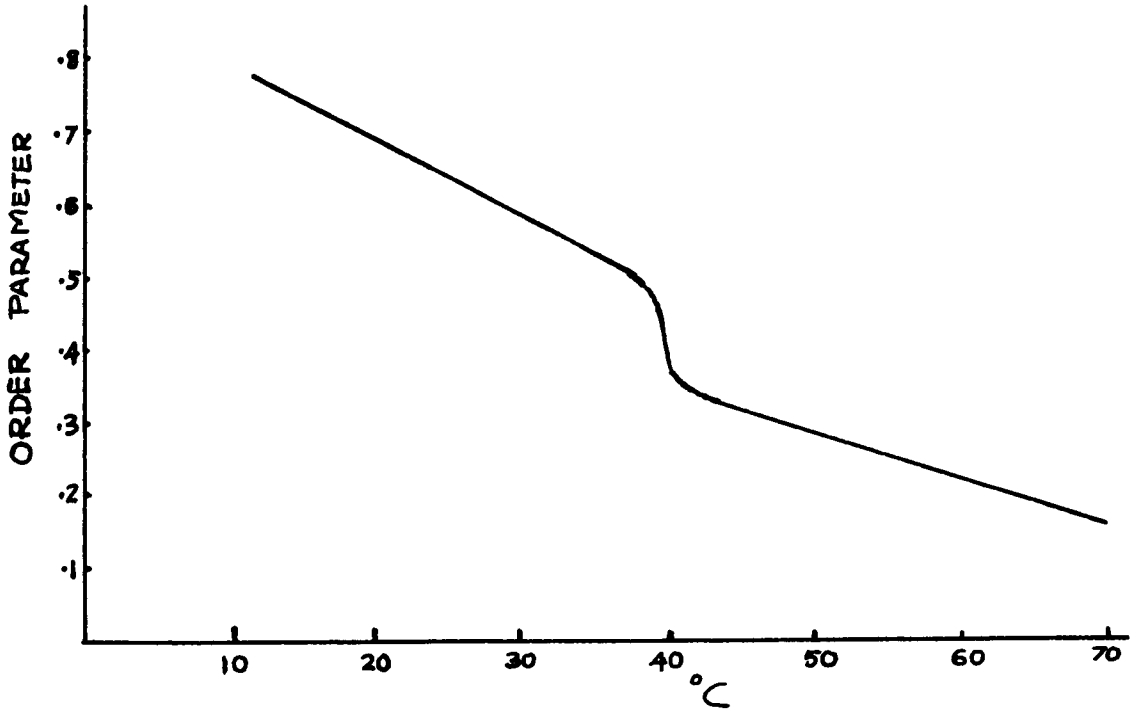
A Plot of order parameter for phospholipid spin label present to the extent of 1 mol % in an aquous dispersion of dipalmitoyl lecithin, as a function of temperature.

B Paramagnetic resonance intensity of 3,4 dihydro-2,2,6,6-tetramethyl piperidine-1-oxyl in the fluid hydrophobic region of dipalmitoyl lecithin.

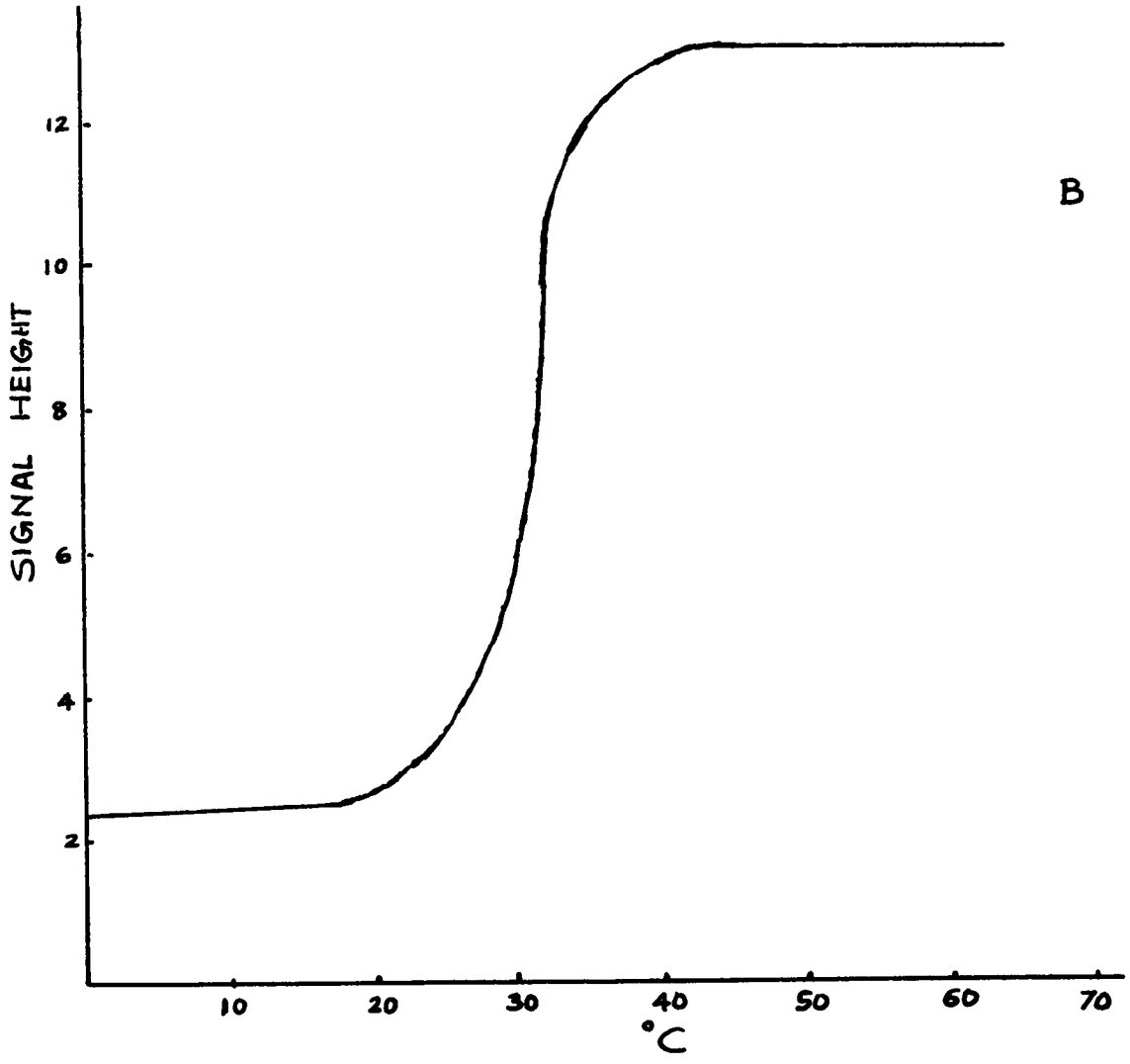
A: figure 7 in reference 45 redrawn

B: figure 9 in reference 45 redrawn

A


ORDER PARAMETER
·8
·7
·6
·5
·4
·3
·2
·1
10
20
30
40
50
60
70
°C

B


SIGNAL HEIGHT
12
10
8
6
4
2
10
20
30
40
50
60
70
°C

out this agreement, as questions have been raised concerning the extent to which a spin label perturbs the bilayers. In the case of lecithin no significant perturbation was found.

Table IX gives the summary of the vibrational modes, the observation, and the important mode characteristics responsible for these observations. The results in Tables III and IV indicate very clearly (refer to figures 19 A and B) that the area of the methylene deformation vibration band (1470 cm^{-1}) remains constant throughout the transition. The half band width, however, is found to increase, thus resulting in the apparent decrease in peak intensity. The plot of the half band width vs. the temperature plotted in figure 19 B resembles closely the plot in figure 18 A and also the plot in reference 45. The reason for the area of the band, at 1470 cm^{-1}, remaining constant through the transition is that the number of oscillators (absorbers) remains the same even though the environment changes during the transition, thus keeping the overall integrated intensity constant. The half band width, however, increases from 24 cm^{-1} to 39 cm^{-1} as the phase transition occurs. The reason for this is the methylene deformation vibration is localized on one carbon and is sensitive to environment. As the temperature increases more and more randomization of the hydrocarbon chains occurs. This randomization changes the environment and

Table IX

Vibrational mode	Observation	Important mode characteristic
C-C stretch (symmetric & asymmetric)	frequency shift	strongly coupled C-C oscillators
C-H stretch (symmetric & asymmetric)	relative intensity change	crystal orientation localized on one carbon
CH_2 deformation	band broadening	localized on one carbon and sensitive to environment

causes the excited state to have a series of closely spaced levels, thus causing the half band width increase and decrease in peak intensity.

The Raman spectra, because of poorer signal-to-noise ratios than infrared spectra, are not useful for measurements of transition temperatures. Nevertheless, a considerable amount of information about the transitions can be gleaned from these spectra. In the infrared spectra only the CH_2 deformation vibrations appear to be sensitive to the thermal phase transitions, whereas in the Raman spectrum changes appear in the C-H stretching and C-C stretching regions as well.

As in the infrared spectra, one finds that all changes in the Raman spectra observed at thermal phase transitions are in bands associated with the methylene groups of the fatty acid chains. No difference appears in the environment of the phosphate or choline methyl groups. This general result is consistent with that of other techniques.

The changes which do appear are relatively easy to understand. As seen in figures 22 and 23, the changes can all be described as relative intensity changes, some involving complete disappearance of one or more bands.

In the C-H stretching region, the initially different relative intensities of two modes, a symmetric and asymmetric stretch (figure22) become almost the same in a

higher temperature phase. These bands appear at approximately the same frequency in crystalline polyethylene, and, as seen in figure 22, undergo a similar change in relative intensity in that case. Although this effect has not been previously discussed, it seems clear that it is associated with a change from an extended, crystalline, methylene chain, to one which is kinked. Further, in molten polyethylene there is considerable motional freedom for the chains. It would not be unexpected for either of these changes to alter the relative intensities of the modes. In both cases the ratio of the polarizability derivatives for different stretches should change. It is not possible to predict, based on our current knowledge of Raman intensities, the direction of this change.

It is interesting that the transition manifests itself here only in intensity and not in frequency shifts. These modes do not involve long range coupling between adjacent methylene groups. Indeed, symmetric and asymmetric motions would appear even in a single CH_2 moiety. Thus the relative intensities of the two bands, which are an average over the whole chain, are sensitive to chain conformation, while the frequencies, which probably reflect very little coupling, remain constant.

The C-C stretching region complements these observations. Again, analogous changes are observed in the lipids to those in polyethylene. This is clearly seen

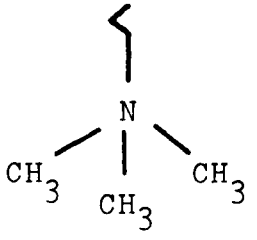
in figure 23. Three bands are observed in the lipid spectra, even at the lower temperatures. These have frequencies of 1130, 1090 and 1060 cm^{-1}. In crystalline polyethylene only two of the bands are found, those at 1130 and 1060 cm^{-1}. These have been assigned as the asymmetric and symmetric C-C stretching, respectively. In molten polyethylene, one sees only a broad C-C stretching band centered near 1080 cm^{-1}. The lipids also show an increase in the intensity of this central band relative to the two side bands. However, the center band is initially more intense in the lipids than in polyethylene, and at least in the LL-water case, there is evidence that the side bands do not completely disappear as they do in polyethylene.

The collapse of symmetric and asymmetric C-C stretches into a central maximum means that an ordered, crystalline chain, has become kinked with a broad distribution of different structures. When this happens the distinction between symmetric and asymmetric stretching is lost.

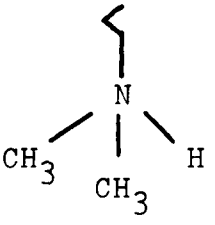
The presence of the band at 1090 cm^{-1} even in the lower temperature phase of the lipids may be evidence for some disorder at these temperatures. However, this band could also arise from a breakdown of translational symmetry selection rules ($k \neq 0$ modes become allowed) which has been previously noted for crystalline alkanes in the literature[76-79]. It is not easy to distinguish between these possibilities.

II Effect of polar head group

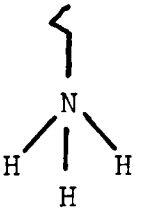
Polar head groups of phospholipids found in nature are found to be wide in variety. In this section, the discussion will be limited to the transition characteristics of the neutral phospholipid gels with the following variation in their polar head group.



gel 1



gel 2



gel 3

The behavior of a ternary mixture gel containing neutral lipids, an anionic phospholipid, and water is considered in a later section.

Except for the gel 3, the other two lipids contained the same two hydrocarbon chains, derived from palmitic acid, attached at the glycerol end, whereas the lipid used to form lipid gel 3 contains two hydrocarbon chains but they were present primarily as a mixture of palmitic and oleic acid esters with small amounts of stearic and linoleic acid chains.

Table X gives the summary of the range of temperature (RT) and mid transition temperature (MT) observed in the endothermic phase transitions of those gels.

Table X

Lipid	# of hydrocarbon chains	# of methyl group at nitrogen of lipid	RT °C	MT °C
Dipalmitoyl lecithin	2	3	43.5-47.5	45.5
L2,3 Dihexadecyl glyceryl NN dimethyl phosphoryl-amine	2	2	44-53	48
Phosphatidyl ethanolamine	2	0	12-18[a]	15[a]

a) Transition temperatures refer to major transition of the system.

Comparison of results obtained for PEA gel with the other two gels becomes difficult because of the differences in the hydrocarbon chains.

The exact nature of configurational freedom of the hydrocarbon chains in such mesophases is not easy to predict. Generally, one can conclude that the polar head group of the lipid has something to do with the configurational freedom of the hydrocarbon chains. The two possibilities are: Either the interaction and the nature at polar head group is responsible for keeping the hydrocarbon chains in liquid like and highly disordered form, or responsible for keeping the hydrocarbon chains in extended and ordered form.

The RT and MT of endothermic phase transitions of gel 1 and gel 2 indicate the following: In the case of gel 2 the nature of configurational freedom of the hydrocarbon chains seems to be a little more extended and ordered than that of gel 1 which is reflected in a slightly higher MT observed for gel 2. Extending this idea one would expect a higher MT ($>48^{\bullet}$) for gel 3. But the comparison becomes difficult because of the structural differences in the hydrocarbon chains of PEA used for making gel 3. No literature value is available for such transition involving lipid gel containing pure dipalmitoyl (C_{16}) phosphatidyl ethanolamine.

Apart from the electrostatic attraction between the water molecule and the polar head group, the one force which seems to be more predominant here is hydrogen bonding.

III Interaction of anionic phospholipid with neutral lipid-water gels as evidenced by the phase diagram, mixed lipid-water gels and freezing point depression calculation using DTA results.

It is well known that the membranes as a macromolecular complex posess cation exchange properties. Nash and Tobias in 1964[61] illustrated the potential importance of the phosphatidyl serine of membranes in relation to calcium ions. The limiting conductivity of the membrane with decreasing ionic strength supported the theory that the membrane containing phosphatidyl serine behaved as a cation exchanger for calcium. Nash and Tobais suggested further from their work that certain physiological properties such as uptake of calcium and high concentration of ions in biological membranes can be attributed to phosphatidyl serine in particular.

The polar head groups tend to react electrostatically with other polar groups. These electrostatic forces may bind polar head groups of lipids to oppositely charged groups, resulting in binary and ternary complexes. The length of the hydrocarbon chain, the number of chains and the charge on the polar head groups affect the transition temperature of the lipid gel formed from them.

Interesting results were obtained by infrared study

of gels formed with the anionic phospholipid phosphatidyl serine. From figure 28 it is clear that even the presence of a small amount of phosphatidyl serine (0.05 mole fraction) depresses the transition temperature by about 20° from that of a pure PC-water gel. DTA results predict a depression of freezing point of such a ternary mixture can only be about 1°. This suggests that the anionic phospholipid structurally interacts with the lecithin-water gels in such a way that the paraffin side chains in the ternary mixtures have become more fluid.

It is of interest to compare this effect on the PC-water transition temperature of an added ionic phospholipid, to that produced by cholesterol. The latter broadens the transition to a very wide temperature range, and also shifts the transition to lower temperature.

As the mole fraction of PS increases from .05 to .18 the transition temperature increases from 20° to 43°. Above this point, however, there appear to be two transitions. This suggests that at large concentration of anionic phospholipid there may be more than two mesomorphic phases coexisting. One may speculate that the higher temperature transition comes from a phase richer in PS, while the lower temperature transition is from a PC rich phase. Ohki has proposed the

existence of such phases in biomembranes in the past[80].

A phase diagram obtained by plotting transition temperature *vs.* mole fraction gives a good picture of the influence of the presence of PS in PC-water mixtures. No experiments were done with mixtures containing more than 0.37 mole fraction PS as all the PS bearing membranes contain much smaller amounts.

IV Protein and lipid complex

Mitochondrial membranes contain phospholipids as their primary structural materials, and contain cytochrome C as a primary functional material. Numerous observations implicate phospholipids in the function of cytochrome C[81]. As with other systems, study of structural features[74] and the nature of model complexes formed between phospholipids and the protein may throw light on the molecular architecture of lipoproteins in those biological membranes.

Many possible mechanisms of complex formation were suggested[65-67, 69]. All of them agree on one point, namely that the linkage between phospholipid and protein is not covalent. Reich and Wainio[65] suggested that primary complexing may involve electrostatic linkages between the phosphoric acid group of the lipid and free amino groups in the protein, with secondary binding occuring through dipole attraction among the phospholipid molecules themselves. This accumulation continues until the complex precipitates from solution, resulting in an insoluble complex. According to Das et al[66] complexes of phosphatidyl ethanolamine and phosphatidyl inositols result from electrostatic bonding between phosphate groups and all or part of the 32 free amino groups of cytochrome C. They also found that lecithin (phosphatidyl choline) complexes primarily to acidic

sites of cytochrome C followed by secondary hydrophobic bonding of additional lecithins. They concluded that the lecithin-cytochrome complex does not involve the amino groups of cytochrome C. Gitler and Montal[69], on the other hand, suggested that phospholipid-protein complexes are probably formed by ion pair interaction between protein cationic groups and lipid anionic groups. Studies with these model systems have been done with both soluble and insoluble complexes (see page102). To our knowledge all of the insoluble complexes studied in the past contained ferricytochrome C.

In this study insoluble complexes were prepared between L 2,3 dipalmitoyl α glyceryl NN dimethyl phosphorylamine and either oxidized or reduced cytochrome C. When 2×10^{-3} m mol of lipid was reacted with 2.5×10^{-5} m mol of cytochrome C in either the oxidized or reduced form, it was found that 43% of the oxidized cytochrome C combined with the lipid to give an insoluble complex whereas only 23% of the reduced cytochrome C combined with the lipid. The molar ratios of lipid : oxidized cytcchrome C and lipid : reduced cytochrome C are 44:1 and 80:1 respectively. The lipid chosen here is neither phosphatidyl ethanolamine nor phosphatidyl choline but rather the homolog with two methyl groups and one hydrogen on the nitrogen of the lipid polar head. One could interpret this result in the manner of reference 65 by saying

that 32 lipids combined with the available 32 anionic positions of the cytochrome C and the remaining 12 lipids combined by a dipole interaction mechanism. Still the question remains to be answered as to why a substantially different number of molecules of lipid combine with 1 molecule of reduced cytochrome C. Although it was reported that the heme iron is not involved in the complexing[65], it appears [71, 82] that the arrangement of the amino acids around the cytochrome C depends on the oxidation state of the heme group. From a study of hydrogen-deuterium exchange of cytochrome C Ulmer and Kagi[82] concluded that the oxidation and reduction of the heme group induced change in the conformation on an appreciable portion of the cytochrome C molecule. This indicates why the availability of anionic sites may alter the approachability of lipid as the oxidation state of the heme group is changed. It is not very clear whether the presence of Na^+ ions in reduced cytochrome C solution was at all responsible for a large number of lipid molecules bonding to one ferrocytochrome C molecule. It is widely believed that unlike divalent cations, monovalent cations do not have any complexing effect other than a salting out effect.

Infrared results reveal interesting information regarding the environment of lipids in both the lipid-ferricytochrome C (O-complex) and lipid-ferrocytochrome

C complexes (R-complex). The pure lipid gel has an ET-OT of 9°. This is a relatively sharp phase transition for these systems. In the R-complex, the lipids are probably in different kinds of environments, making the phase transition broader, with an ET-OT of 21°. The lipids in the O-complex seem to exist in a more uniform environment resulting in a very sharp transition with ET-OT of 6°. The O-complex thus has a sharper transition than the transition of the pure lipid gel. This indicates that all the 44 lipids are organized around one ferricytochrome C molecule in a single and well defined phase.

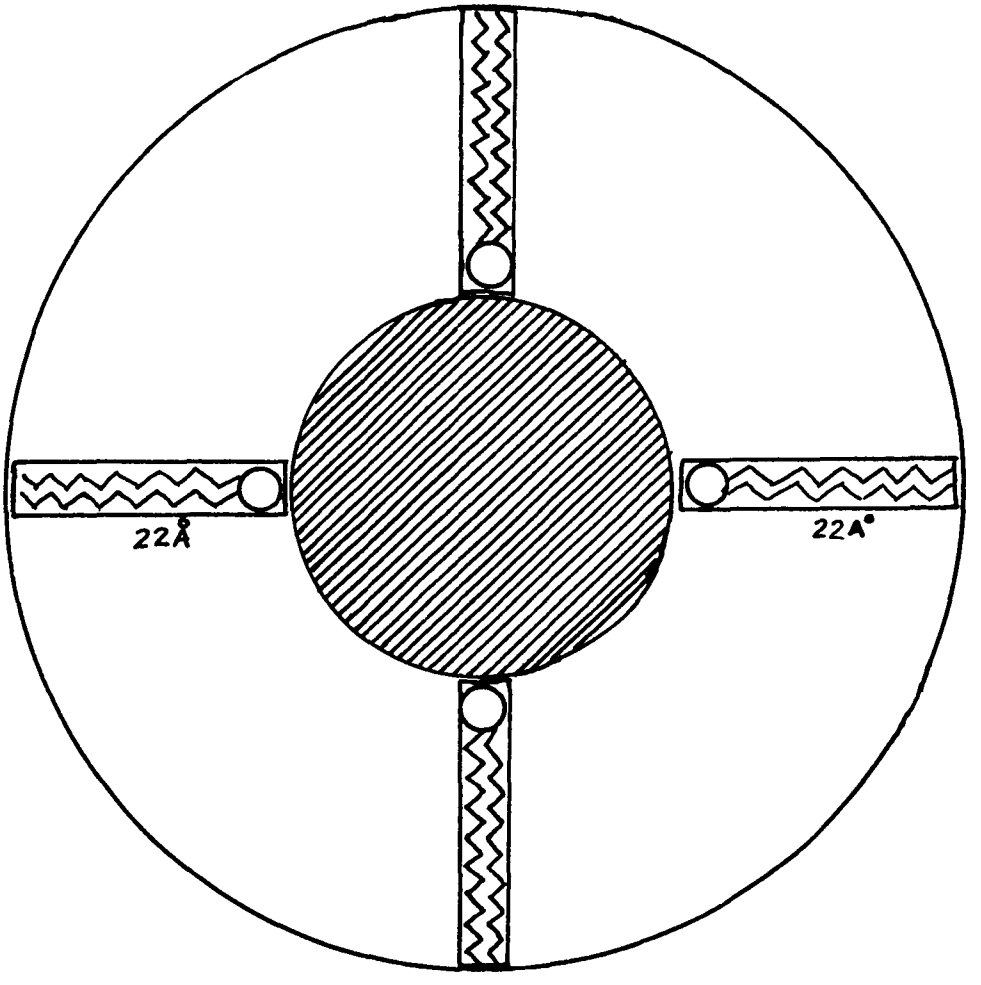
This does not seem to be true with the R-complex, which shows a very broad transition. It appears that in the R-complex the lipids are bonded to ferrocytochrome C not only on primary bonding sites, but also by a secondary type of bonding, either hydrophobic or dipolar. This conclusion about the broad temperature range of the transition is consistent with the large number of bound lipid molecules in this complex.

The MT of the pure lipid gel is 48°. The MT of the O-complex is 51° and the MT of the R-complex is 53.5°. Both the O-complex and the R-complex have a higher MT than the pure lipid gel. This indicates that in both the O-complex and R-complex the lipids are organized in a distinct arrangement which is thermo-

Figure 39

Sketch of lipids packed around a cytochrome C molecule (cross sectional view)

At the centre one cytochrome C molecule is located, having diameter of 31 Å. Surrounding the cytochrome C molecule, lipid cylinders are arranged, (length 22 Å and diameter 10 Å).


22Å
22Å

dynamically different from that in the pure lipid gel.

The MT of the R-complex is 2.5° higher than that of the O-complex. This may be due to a slight inhibition of the molecular motion of some of the hydrocarbon chains of the lipids in the R-complex, thus resulting in a slightly higher transition temperature compared to the O-complex.

A simple but interesting calculation can be carried out by taking the diameter of a cytochrome C molecule as 31 Å[74] and assuming lipid as cylinders of length 22 Å and 10 Å diameter. This reveals that as many as 90 lipids can be packed around one cytochrome C molecule in the available annular volume. This is shown in figure 39.

V Results from DTA and light scattering

It is interesting to note that both infrared and DTA point to the fact that the endothermic phase transition is sensitive to the type of polar heads. Although the transition temperatures from infrared and DTA experiments do not agree numerically, they do agree in the trend (see figures 33 and 34). For example, a PC-gel with three methyl groups at the nitrogen of the lipid polar head shows a slightly lower transition than that of the lipid gel with two methyl groups at the nitrogen of the lipid polar head (refer and compare figures 33 A and B, 18 A and 32 B). Both of these transitions appear to be sharp in both DTA and infrared studies indicating the presence of well defined phases. The LL, which contains only one hydrocarbon chain but contains three methyl groups at nitrogen of the lipid, forms a gel with water and shows a slightly broader transition in both DTA and infrared studies (figures 33 C and 18 B). It appears that the sensitivity of these mesomorphic phase transitions is more accented in the infrared than in the DTA. This is because in infrared the observations are made on a specific portion of the molecule (methylene deformation vibration of the hydrocarbon chains of lipid tails) while following the phase changes. From the thermogram one could obtain the thermodynamic quantities for these phase transitions whereas

The infrared measurements can give information on the environment and state of lipids in these mesomorphic phases.

Light transmission studies of the lipid gel indicate that during the phase transition the hydrocarbon chains of the lipid become more transparent to light (figure 35 a and b). This transparency to light is accented by inserting a micro glass rod in the capillary tube, which reduces the thickness of these lipid gels.

Light scattering studies of the lipid gels with the help of a polarizing microscope give the pictures shown in figure 36 A-D. One could observe a change in the depth of color in these photographs as the lipid gel is undergoing the transition. This is attributed to birefringence arising from the optical anisotrophy of the molecules. Due to thermal energy the molecules tend to orient in specific directions. During transition from crystalline to liquid crystalline phases, different parts of the lipid are in relative motion with respect to one another. The system as a whole exhibits birefringence. This type of birefringence or dichroism is observed in chloroplasts[84], myelin sheath[85] and other biological membranes.

VI Hysteresis and cooperativity

Previous sections on infrared and Raman results on neutral lipid gels, mixed neutral lipid gel, anionic lipid gel and lipid-protein complexes discussed the phase transition of part of a single molecule (melting of the hydrocarbon chain). In this section the discussion centers around the aspect of transition relating to the collection of molecules, the lipid bilayer as a whole.

A lipid gel (Lipid: synthetic lecithin) was heated through the phase transition and cooled back. The plot of relative absorbance vs. temperature gives the hysteresis type plot shown in figure 32 D. This observation of hysteresis implies a kind of cooperative phase transition.

What does cooperative mean for such a transition? As the lipid gel is being heated the thermal perturbation causes disorder of a chain on a molecule. This disorder created by the thermal perturbation is like a lattice defect in crystals. The effect of the defect propagates through the bilayer. The net result is a cooperative phase transition. Hysteresis occurs because to reverse the phase transition, liquid crystalline to crystalline, and return to an ordered phase work must be done. Work done is proportional to the area between the curves.

Thus the cooling and heating curves have the same shape (ET-OT = 12° for both these curves), but they have different MT. MT of the heating curve is ~5° higher than the MT of the cooling curve. DTA cannot make such measurements on shape differences on heating and cooling.

There are other evidences in this thesis for this cooperativity. The effect of polar head groups on the phase transition is one thing to note. Here even a small change in the polar head groups affect transition temperatures. As the number of methyl group attached to nitrogen of the lipid polar head is reduced from 3 to 2 the phase transition temperature changes. This may be attributed to the slight differences in the spacing of the hydrocarbon chains of the lipids.

Other evidence comes from the study of the cytochrome C-lipid complexes. Both the R-complex and O-complex give a slightly different transition temperature from that of the pure lipid gel. This implies that the differences in the packing of the lipids around the ferro and ferricytochrome C molecule affects the hydrocarbon chain melting.

The hysteresis effect illustrates the long range multimolecular interactions existing in these lipid bilayers.

CONCLUSION

Earlier study[54] of model membranes was limited to the study of melting of anhydrous phospholipids using infrared spectroscopy. In this work infrared and Raman spectroscopy have been used extensively as successful tools for measuring the phase transition of model membrane systems. In both these techniques, the number of bands observed, their relative intensities, and their band widths, all yielded information on the mobility of the hydrocarbon chains of the lipid phase. Degree of fluidity of the hydrocarbon chains of the lipid tails seems to be a requirement for proper membrane function.

Influence of variation of charge at the polar head of the lipids on the temperature of the hydrocarbon chain transition of the lipids has been studied by the infrared technique by the addition of anionic phospholipid to the neutral lipid gel. The presence of a small amount of anionic lipid was found to lower the transition of neutral lipid gel to a great extent. This reveals the relationship between the charge on the polar head and the mobility of the lipid tails. This relates to membranes bearing only a small amount of phosphatidyl serine (anionic lipid) which are responsible for active transport and exchange of ions.

Infrared results agree quite closely with the

results of electron spin resonance study of spin labelled lipid gels. This agreement indicates not only the importance and usefulness of the infrared technique for the study of membranes and model membranes, but also the small perturbing influence of the spin labels on the phase transition studies.

Infrared and Raman techniques, having a time scale of 10^{-12} to 10^{-14} second, have been used here successfully to study fast molecular motions involving the hydrocarbon chains of lipid tails.

Study of protein-lipid complexes by infrared spectroscopy revealed a slightly different kind of packing existing in the ferricytochrome C-lipid complex than in the ferrocytochrome C-lipid complex. The hysteresis effect observed in the lipid gel is further evidence for cooperativity of these lipids in the phase transition. This leaves open to question the primary electrostatic bonding and secondary type of bonding (either dipolar or hydrophobic) suggested by previous workers[65, 66]. More work is needed to clarify the type of bonding existing in these biologically important macromolecular complexes.

This study proves that by infrared and Raman studies one could obtain reasonable information on the phase transition temperature, the number of transitions, and

to a certain extent the characteristics of the meso-
morphic phase transition of membranes and model systems.

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