BIOPHYSICAL STUDIES ON NATURAL AND ARTIFICIAL MEMBRANES

PROJECT REPORT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE M. Sc. DEGREE.

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ABSTRACT

The effect of temperature on the absorption [illegible] characteristics of intrinsic probe, chlorophyll, in case of chloroplast, and fluorescence characteristics of extrinsic probe, ANS, bound to liposomes have been investigated in this study. The effect of temperature on fluorescence characteristics of chlorophyll, *in vivo* (chloroplast membrane) (Murata, 1975) and in liposomes (Calbow, 1973) have been studied, but there has been no study so far, on the absorption characteristics of chlorophyll as a function of temperature.

The difference spectrum, upon cooling of the sample ($22^{o}C \longrightarrow 11.4^{o}C$) reveals an increase in absorption of chlorophyll at 410, 440, 470, 500 and 675 nm and decrease at 690 nm. As the chloroplasts are heated from $11.4^{o}C$ to $29.5^{o}C$ ($22^{o}C \longrightarrow 11.4^{o}C \longrightarrow 29.5^{o}C$), a decrease in absorption at 410, 440, 470, 500 and 675 plus an increase at 690 nm is observed and upon further heating upto $42.04^{o}C$, the absorption at these wavelengths decreases further significantly. The chloroplast, upon cooling to $13.6^{o}C$ ($42.04^{o}C \longrightarrow 13.6^{o}C$) do not show the similar absorption profile when compared with either 11.4 or 15.9C of cooling $\longrightarrow$ heating cycle.

The spectral changes can be attributed to the state of aggregation of the chlorophylls as a result of a change in the phase of membrane lipids (solid $\longrightarrow$ liquid crystalline state and vice versa) or change in the orientation of the chromophore resulting in a change in the electronic state function.

In model membrane system, the hydrophobic probe, ANS, is used to monitor the phase transition in liposomes and lipid dispersions of egg lecithin. A phase transition at $44.5^{\circ}C$ is quite sharp in liposomes prepared under N_2-atmosphere. The sharpness of phase transition is reduced when it is observed either in PC (phosphatidyl choline) dispersions or in liposomes prepared under Non-N_2-atmosphere.

BLM's stability was checked and it was stable for 15 min in our preparation.

INTRODUCTION

(1) General:

Structure and functions of biological membranes have been and are the subject of intense and broad scale investigations. The interest in this field has gained momentum in past two decades with the increasing awareness that membranes perform many functions (see Table 1) in addition to partitioning of cellular compartments and mediation of transport between them.

It is well established fact that most of the biochemical and biophysical functions are related with the conformation of membrane. The diversity of membrane associated phenamena is reflected in the membrane composition, namely types of lipids and proteins, and ratio between proteins and lipids. It has also been observed that the lipid constituents of membranes from green plants adapted to the temperatures mainly consists of unsaturated fatty acids (Lehninger, 1975; Murata, 1975). Unsaturated fatty acids have lower melting points than saturated fatty acids of same chain length. The composition is very much related to the phase transition phenamena which results in drastic changes inthe functioning of biomembranes.

The functions of membranes in various kinds of cells as given in Table 1 are the function of interaction between its constituents and interactions with its surroundings, both internal and external. In intact natural membrane systems, metabolic status (proton and salt concentration etc) is variable, and due to the complex composition of membranes their responses to the surroundings are of complex nature. Therefore, to study the effects of different parameters, in systematic way the model membranes are chosen to get some insight into the structure and functioning of biomembranes. The basic question to

TABLE - 1

SOME FUNCTIONS OF BIOLOGICAL MEMBRANES*

Membrane Type	Function
All plasma membranes	Compartmentalization, maintenance of osmotic balance, general and selective diffusion of metabolites and ions, and their physiological regulation; active transport, phagocytosis, pinocytosis and secretion
	Cell adhesion, aggregation; specificity and plasticity of membrane contact.
	Motility, cytoplasmic streaming, coalescence, contraction and expansion; translational and rotational movements of membrane-bound organelles.
	Receptor for surface antigens, hormones, transmitters and other biodynamic substances.
	Limitation of organ growth; impaling and conveyance of cytoplasmic and luminal matrix; reversible geometric transformations between various forms, such as spherical, tubular; transformations from elastic to rigid forms, and vice versa. Formation of tissue-specific patterns wit specialized functions (brain).
	Differentiation and dedifferentiation; DNA replication and RNA stabilization.
Myelin	Electrical insulation
Nerve plasma membrane	Generation and conduction of nervous impulse.
Sensory neurons	Sensory transduction
Chloroplast and mitochondrial membranes	Photo-and oxidative phosphorylation-energy transduction in general.

* Taken from Jain (1972).

which studies of physical properties of model systems are addressed to, is to have knowledge about the relationship between structure and certain functions of lipid component in natural membranes. In order to answer this question, it is quite relevant to investigate the structure and composition and properties of model membranes.

In this study we have tried fist to characterise the model membranes (BLM & Liposomes). We have made phase transition studies both in liposomes and chloroplast membranes. Before presenting our data we would like to discuss first the membrane structure ofthe chloroplast and the model membranes.

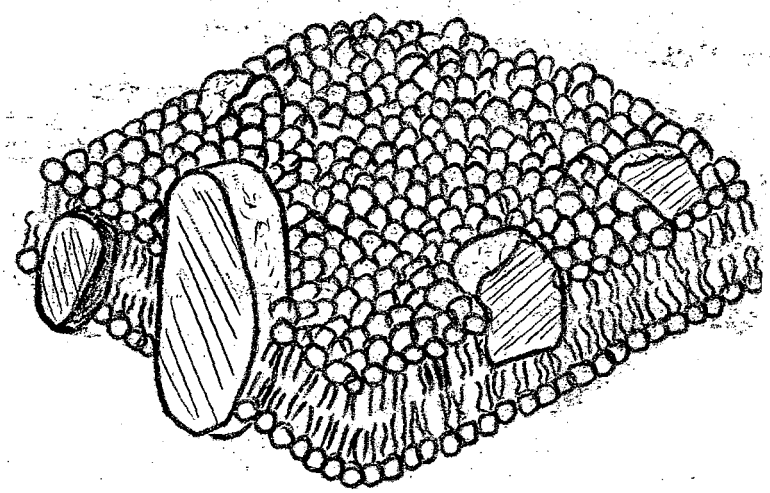
(2) Membrane structure:

Various models have been suggested to describe the structure of biomembranes. A widely accepted general model for membrane structure is the bimolecular lipid leaflet flanked on either side by protein layers. Gorter and Grendel (1925) estimated that area covered by lipids extracted from erythrocytes was about twice the surface area of the cells showing that it has bilayer structure. Some years later this hypothesis was modified and refined into unit membrane hypothesis (Robertson, 1960).

The most satisfactory model of membrane structure to date appears to be 'fluid mosaic model' postulated by Singer and Nicolson (1972), (Fig 1). This model postulates that the phospholipids of membranes are arranged in a bilayer to form a fluid, liquid crystallin matrix or core. In this bilayer, lipid molecule can move laterally, endowing the bilayer with fluidity, flexibility and characteristically high electrical resistance and relative impermeability to highly

Figure 1 :

The lipid-globular protein mosaic model with a lipid matrix (the fluid mosaic model). Schematic three dimensional view. The solid bodies with stippled surfaces represent the globular integral proteins, which at a long range are randomly distributed in the plane of membrane. At short range, some may form specific aggregates, as shown (Singer and Nicolson, 1972).



The Fluid Mosaic Model

polar molecules. The fluid mosaic model postulates that proteins are globular, to account for their high content of α helix. Some of the proteins are partially embedded in the membrane, penetrating into the lipid phase from either sides and other span the membrane.

(2)(i) Chloroplast membranes:

Chloroplasts (Fig 2) are surrounded by two unit membranes, the inner one of which may invaginate to form complex internal lamellar system. Chloroplast membranes are 52% lipid and 48% protein (Mobel, 1970). Much of the chlorophylls and other photosynthetic pigments appear to be bound to the membrane proteins and lipids by hydrophobic forces. Also, the enzymes and other components involved with photosynthetic electron transport are located in chloroplast lamellar membranes. The major lipids are digalactosyl glyceride and monogalactosyl diglyceride. Phospholipids make up 75% of total lipids. The remaining 25% are chlorophylls; Carotenoids and other pigments. Sterol and glycerides are very minor constituents (Tein, 1970)

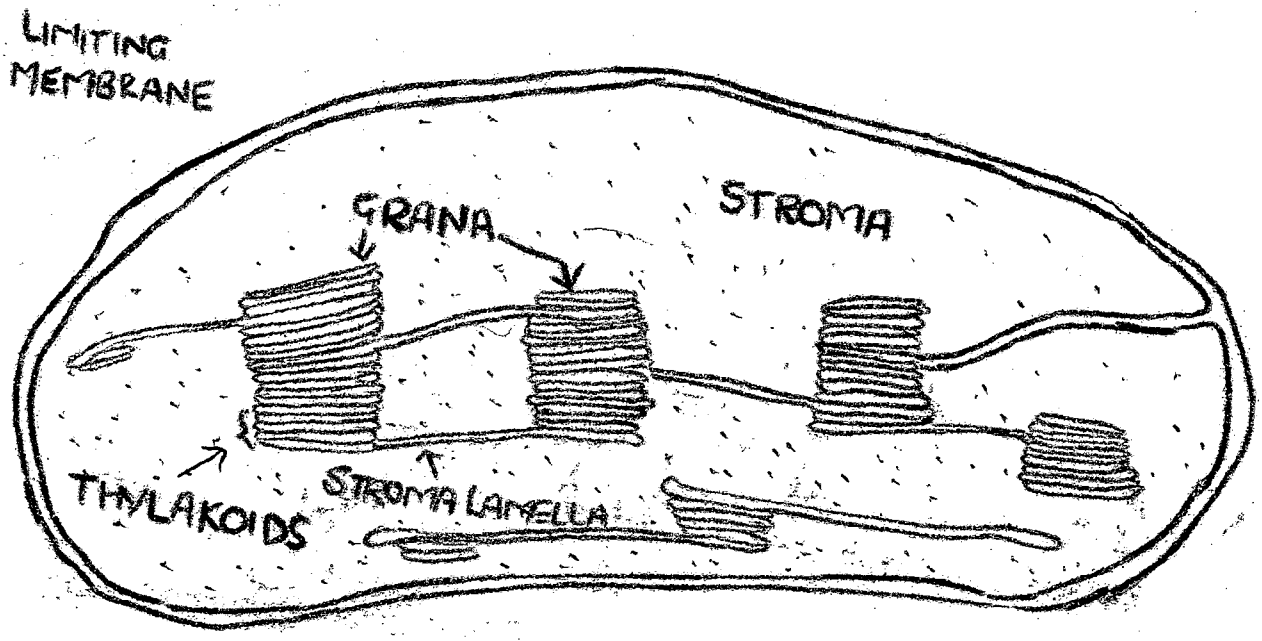
(2)(ii) Model membrane systems:

The idea that isolated and purified phospholipids of cellular origin could spontaneously reform, in presence of water, into a pattern of closed membranes systems (Bangham et al, 1974), probably emerged during the course of andelectron microscopic study that Horne and Bangham (1964) carried out during 1961-1963 shortly after the postulation of unit membrane hypothesis (Robertson, 1960).

Membrane molecules are characteristically amphiphile, that is to say that theyare sufficiently large for different regions of each molecule to behave, so to speak, inda discrete fashion (Bangham, 1978). Typical examples of membrane compounds that exhibit amphiphile

Figure 2 :

Generalised chloroplast from a leaf mesophyll cell (Nobel, 1970).


LIMITING MEMBRANE
GRANA
STROMA
THYLAKOIDS
STROMA LAMELLA

properties to the extent that they form ordered phases in equilibrium with water phase (liposomes) are class II polar lipids (Small, 1970) eg. phosphatedyl cholines, ethanolamines or serines, sphingomyelins, cardiolipins plasmalogens, phosphatidic acids; and cerebrosides and cholesterol.

These compounds when confronted with an encroaching aqueous environment undergo a sequence of assemblages that reflect the thermodynamic perturbations of increasing water- water, water oil and oil-oil interactions. These quasi equilibrium structures should more correctly be termed as smectic mesophases (Bangham, 1978).

In recent two years two types of model systems have been developed both of which are comprised of the lipid component of natural membranes, and in addition, have transverse dimensions similar to that of biological systems. The orientation of lipid component in both of the models is the bimolecular lamellar arrangement suggested by Dayson and Danielli (1952) to be structural form of the lipid component of biological membranes. The physical properties of one model system,(the phospholipid bilayer membrane separating two aqueous phases) have proven to be strikingly similar to the corresponding properties of natural membranes (Hueng and Thomson, 1965, 1966). The other model system consisting of an aqueous dispersions of liquid crystals of phospholipid has been shown to have permeabilities properties similar to those of biological membranes and to be particularly well adapted to study of interactions between phospholipid lamellae and proteins in aqueous systems (Litman and Thomson, 1967).

(2) (ii) BLM- A model membrane system:

When an amphipath above the transition temperature is smeared

across a small hole joining two aqueous compartments, a bimolecular film that separate the two compartments is formed. The only pre-requisite for a membrane forming solution is that an amphipathic compound (eg. phospholipid) and a neutral hydrocarbon solvent be present (Lenaz, G. 1977). Mueller et al, (1962) observed that a drop of solution, placed at an aperture in a teflon septum immersed in a electrolyte solution, rapidly thins and forms a stable membrane. This membrane appears dull grey in color when the light reflected from it, is viewed through a microscope (Fig 3) and hence the name balck lipid membrane.

(2) (ii)(b) Liposomes- A model membrane system:

When the amphipathic lipids are allowed to swell in water above the transition temperature, they form a closed onion like structures (liposomes) that consists of stacked lamellae of phospho-lipids in bilayer configuration with water filling spaces between the lamellae (Fig 4.a). Liposomes have advantages over BLMs, since they are better defined chemically involving no organic solvent and easier to handle. There are two techniques to prepare liposomes.

(a) With sonication (Huang, 1969)

(b) Without sonication (Batzri and Korn, 1973).

Since we used sonication method, here we will discuss about (a) only. Ultrasonic irradiation of liposomes breaks up the coarse particles into small vesicles (Fig 4.b) of 200-500A^{0} diameter (Lenaz, G. 1977). Optical methods can be applied to them, in studying osmotic behavior and structural changes as a functions of physical parameters; it can be used for study of water permeability, and diffusion rates for ions through phospholipid vesicles (Bangham et al,

Figure 3 :

Diagrammatic representation of formation of BLM by the thinning of lipid film to a bilayer (Jain, 1972).

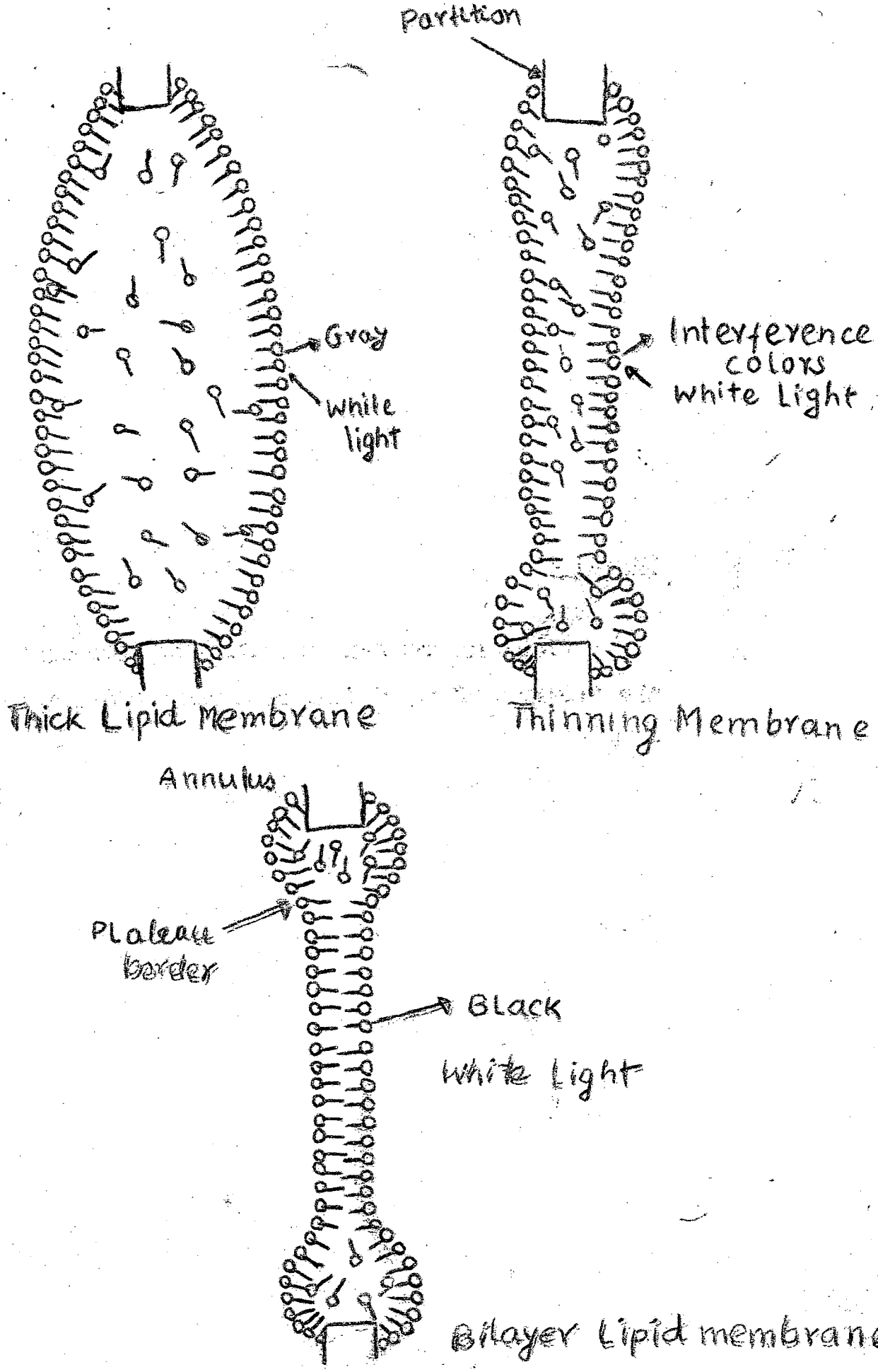
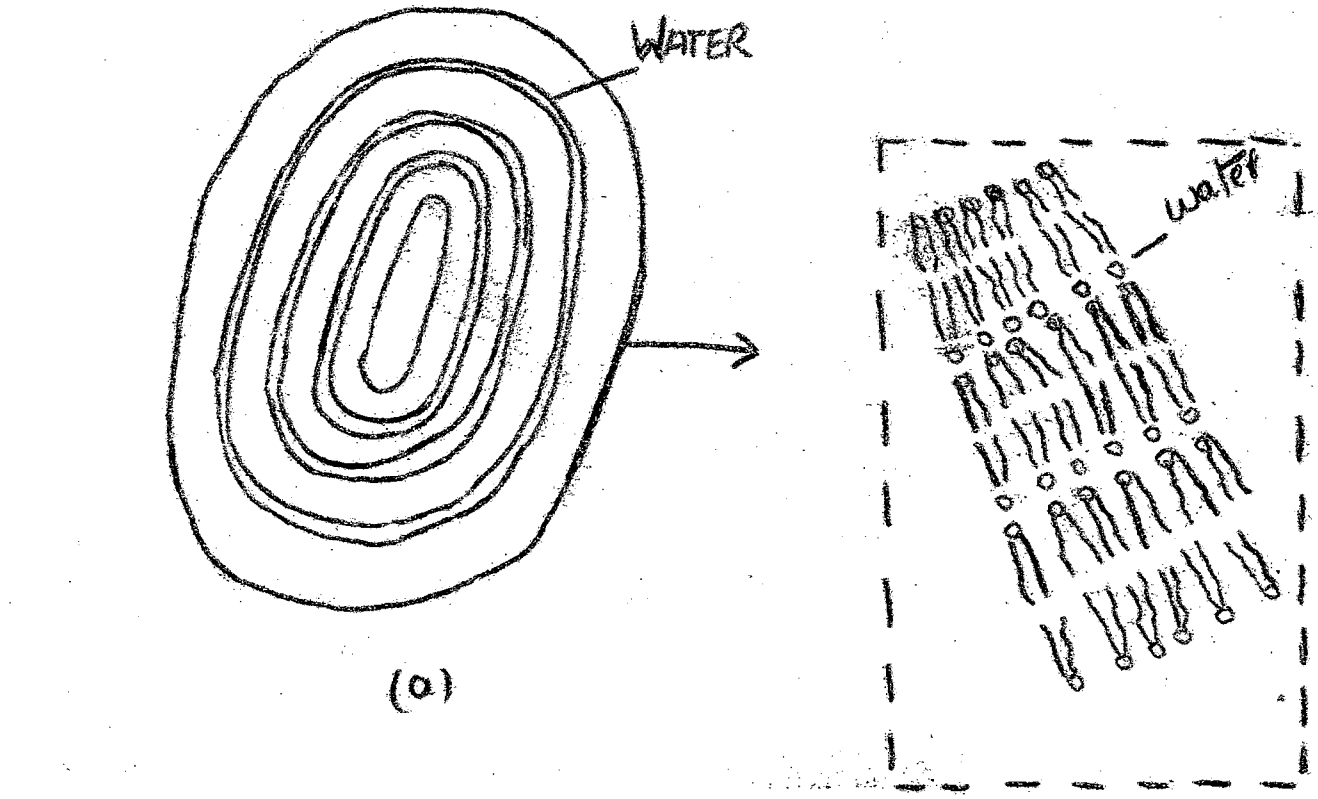

Partition
Gray
white light
Interference colors white Light
Thick Lipid Membrane
Thinning Membrane
Annulus
Plateau border
Black
White Light
Bilayer Lipid membrane

Figure 4:

Structure of liposomes

(a) Multibilayer

(b) Monobilayer vesicle.


WATER
water
(a)
(b)

1965). But there are several disadvantages in preparative methods of liposomes by sonication as high energy sonication often causes oxidation and degradation of phospholipid, therefore, preparation of liposomes without sonication has advantages over ultrasonication procedure (Brunner et al, 1976).

Two model systems are complementary and perhaps the best approach is to carry out studies concurrently.

(3) Phase transitions:

(i) In lipid bilayers: Raising the temperature of a pure crystalline lipid induces an endothermic transition at a specific temperature, where the hydrocarbon chains of component fatty acids melt and become liquid like in mobility.

Artifical as well as certain biological bilayer membranes undergo reversible transitions from an ordered, quasi crystalline to a disordered, liquid crystalline state at a characteristic critical temperatures, T_c. Raising the temperature of membrane lipids to or beyond critical phase transition temperature brings about changes in packing arrangements, mobility and bilayer permeability (Fig. 5). The hydration of polar groups and the degree of incorporation of external compounds are also increased above T_c (Sandermann, Jr. (1978)). These values also depend on the state of ionization and hydration of phospholipid polar head groups. T_c values may be modified by incorporation of foreign compounds eg. cholesterol (Kruyff, et al, 1973), or by binding of divalent cations or proteins or by changes in pH or ionic strength.

(3) (ii) In chloroplast membrane: Instead of fluorescence (Murata, 1975) we have tried to use absorption of chloroplast as a tool to determine

Figure 5 (i):

Change in packing arrangements of phospholipid bilayer ~~or~~/after transition.

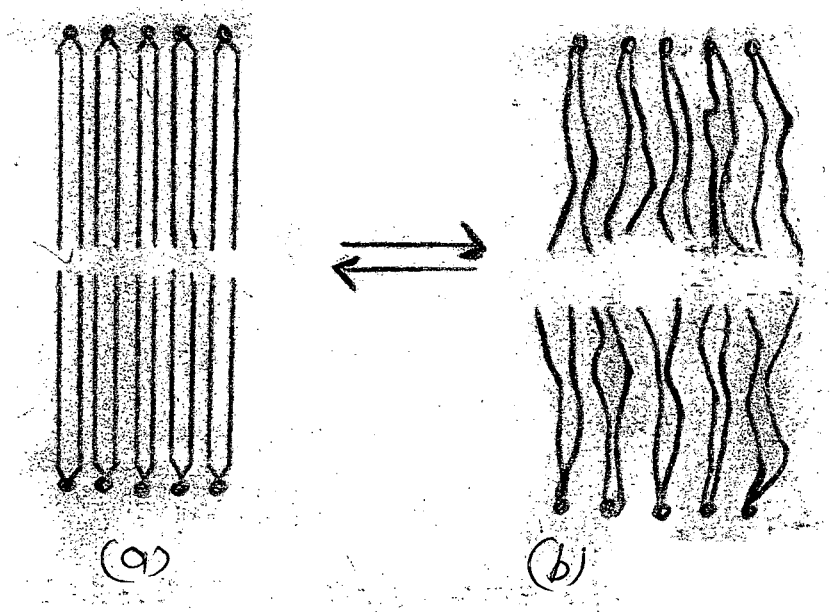
(a) quasicrystalline structure

(b) liquid crystalline

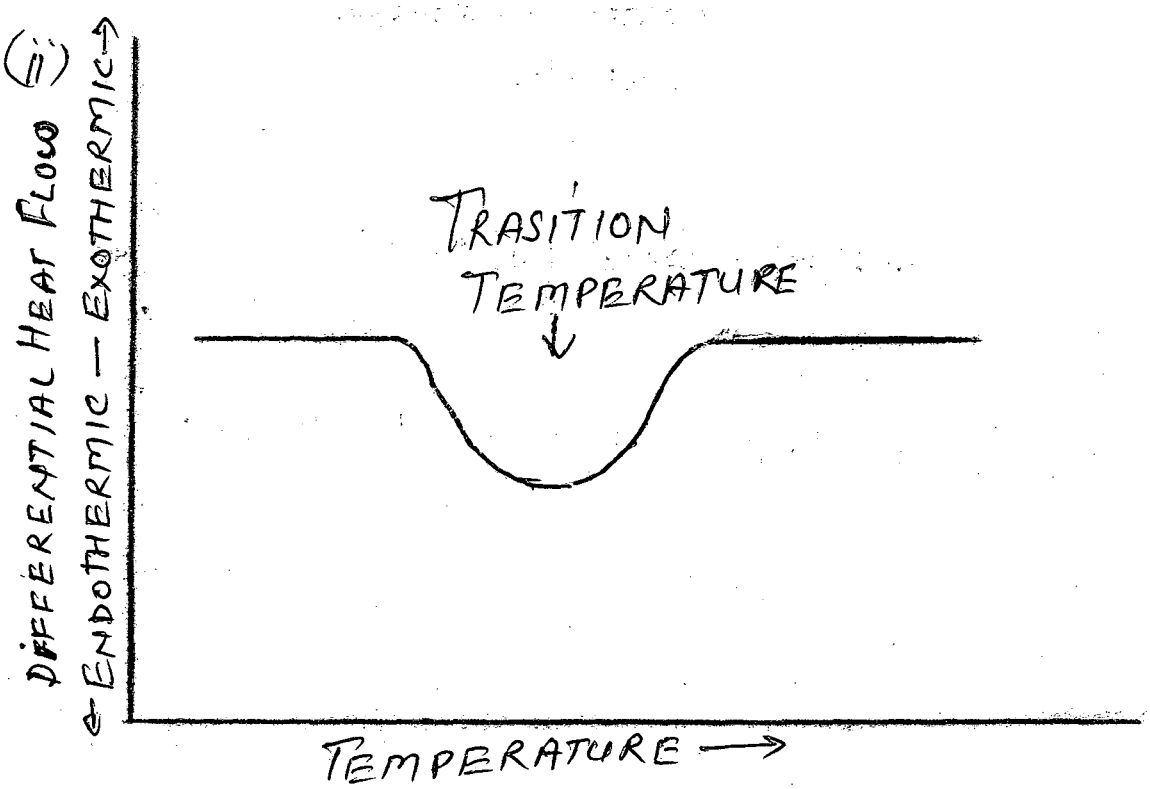
Fig.5. (ii):

Endothermic transition.

(i)


(a)
(b)

(ii)


DIFFERENTIAL HEAT FLOW
← ENDOTHERMIC — EXOTHERMIC →
TRASITION
TEMPERATURE
TEMPERATURE →

the phase transition of chloroplast membranes.

It is known that the temperature of phase transition is lower with a lower degree of saturation of fatty acid chains (Melton, et al, 1964). Biomembranes have high concentration of poly unsaturated fatty acids so that the lipids are primarily in the liquid crystal phase at room temperature (Lee, 1975). There can be a temperature range where regions of membrane, separated laterally, are in solid and liquid crystal states (Murata, 1975).

The decrease in fluorescence of Chl with the change in state of lipid from solid (low temperature) to liquid crystalline state (high temperature) is interpreted, in liposomes, as a result of the formation of aggregates in liquid crystal state(Lee, 1975). A model has been proposed for increase in solubility of Chl with increase in temperature and resulting in the formation of more aggregates (Murata, 1975).

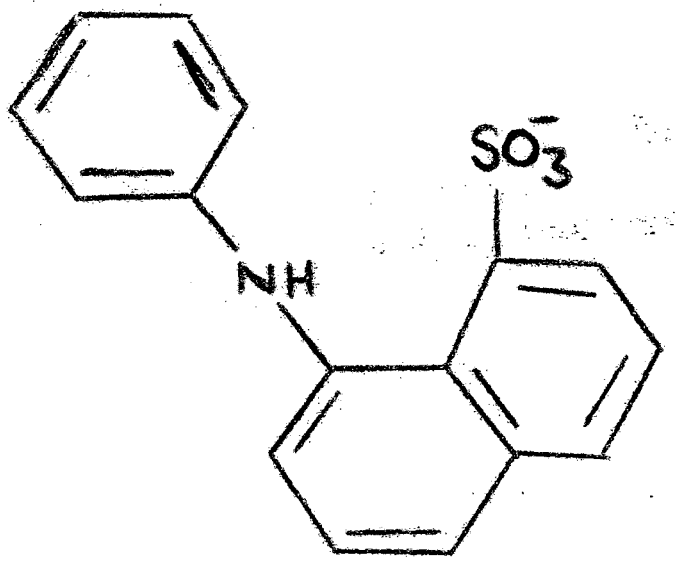
The absorption spectra of photosynthetic pigments in chloroplast membrane, as a function of temperature is described in the present work. Spectral changes are determined by measuring the difference in absorption between two identical samples at different temperatures.

(4) (iii) In model membranes: The determination of phase transition in liposomes was studied by incorporating external fluorescent probes. It is well known that external fluorescent probe reflect the polarity viscosity and other features of their immediate environment (Arindum Sen, 1977).

In our study we have used 1-anilinonaphthalene-8-sulphonate (ANS)(Fig 6) as hydrophobic fluorescent probe.

Figure 6 :

Structure of AIS.



1-Anilino-8-napthalene Sulphonate

(ANS)

(4) Present work:

The complex functioning of biomembranes with simply two main constituents (namely protein & lipids) involves varieties of interactions- electrostatic, hydrophobic, vanderwaal's between water-lipid, water-protein, lipid-lipid, lipid-protein and protein-protein, resulting in a specific conformation to cope with. Any change in conformation of membrane constituents will affect these interactions and viceversa.

Our aim in this work, has been to study the effect of temperature on membrane conformation in model as well as natural membrane systems, and prep-
The parameters that we have studied as a function of temperature are:

1. Absorption properties of chlorophyll in broken chloroplast membrane fragments.
2. Fluorescence properties of ANS bound to liposomes.

It is expected that these studies will help in better understanding of the properties of biomembranes as a function of chemical and physical parameters.

MATERIALS AND METHODS

A. Natural membrane system:

1. Chloroplast extraction.
2. Temperature induced phase transition and absorption measurements.

B. Model membrane system:

1. BLM formation.
2. Preparation of liposomes.
3. Fluorescence measurements

(i) Instrumentation
(ii) Measurements
(iii) Temperature induced phase transition and fluorescence measurements.

A 1. Chloroplast extraction:

Young barley leaves were collected from mature barley plants from fields and chopped into 1 cm long pieces. The leaves were crushed in waring blender for 20 sec with 0.4M sucrose, 10mM phosphate buffer pH 7.8, then squeezed through 8 layered cheese cloth and centrifuged at 500 g for 2 min to remove cell debris. After removing pellet it was again centrifuged at 1000 g for 10 min in K-24 centrifuging machine. Pellet was resuspended in $PO_4^{'''}$ buffer and suspension was taken as sample.

A 2. Temperature induced phase transition and absorption measuremen

Absorption measurements were done with Shimadzu (MPS-5000). Two cuvettes having identical samples were put each in reference and

sample cuvette holders. One sample was cooled or heated by circulating water from a thermostated bath through the cuvetter holder. The rate of heating the chloroplast suspension was 0.5°C/min. A copper- constantan thermo couple was used to measure the temperature immersing it in suspension of sample compartment. The other end of thermo couple was attached to x-y recorder (Rácken Denshi, Japan).

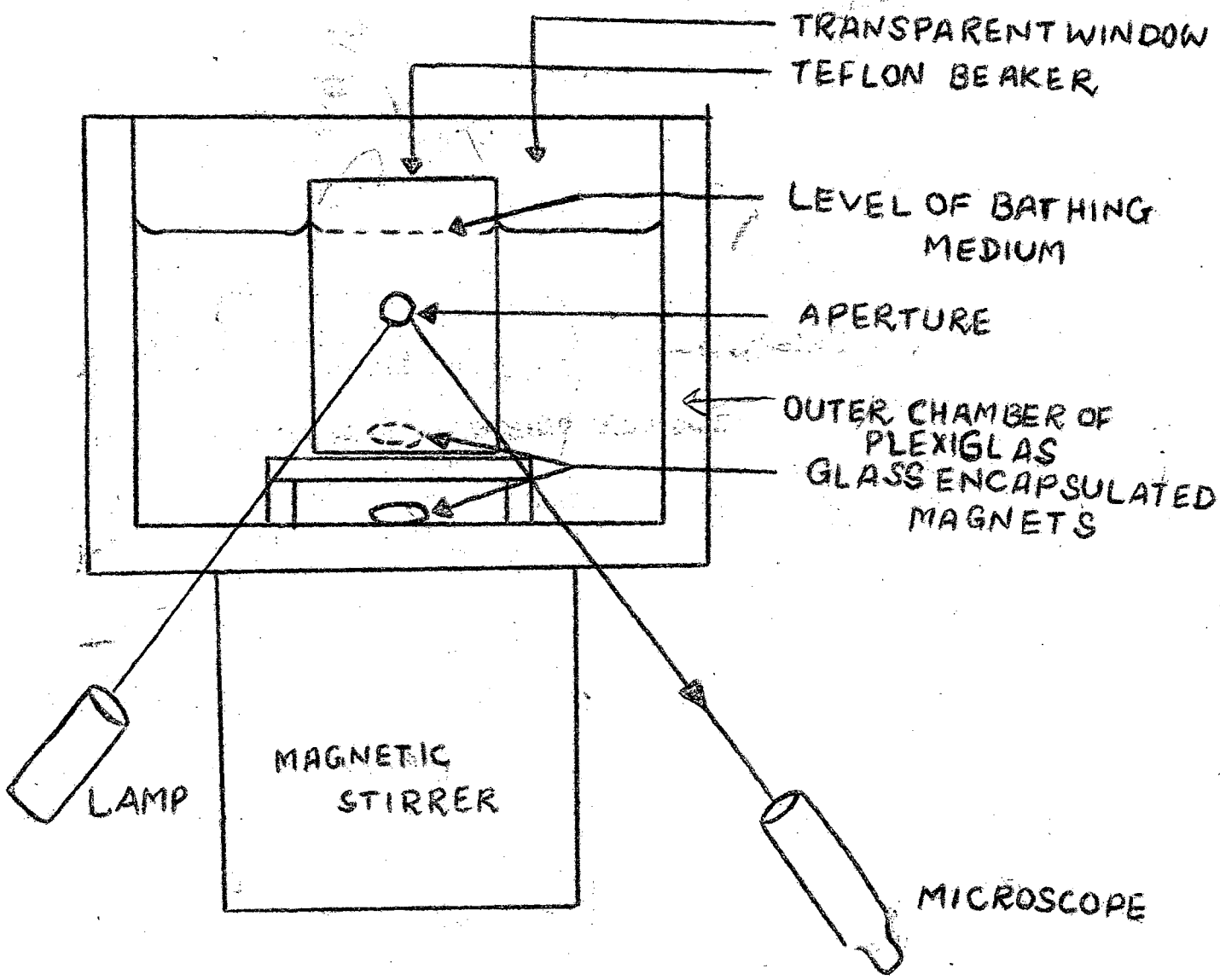
The initial difference spectrum at room temp (22°C) was considered as baseline. Firstly the sample was cooled to 11.4° and while cooling at 12.5°C one difference spectrum was measured. After measuring difference spectrum at 11.4°C, sample was heated upto 42.04°C and during this difference spectra were measured at 15.9°C, 19.9°C, 25°C, 29.5 C, 33.5°C and 42.04°C. Then we started it cooling and during which difference spectra were measured at 36.4°C, 31.25°C, 26.1°C, 26.1°C, 14.8°C and 13.6°C. To determine the Δ O.D. all spectral were set equal at 730 nm where there is essentially no absorption. Difference spectra were measured either on Δ O.D. scale $\pm$ .05 or .01.

B 1. BLM formation:

The experimental set up for obtaining BLM are shown in Fig 7. Before the preparation of BLM, washing of outer chamber (of plexiglas) and inner Teflon beaker (8 ml in volume) were thoroughly washed. For outer chamber washing, it was kept in soap solution for 24 hrs. The teflon beaker was cleaned before use by the procedure by Jain et al.,(1972). Teflon beaker was boiled in 1M KOH solution for 1½ hrs to remove any oily material

Figure 1:

Schematic diagram of ~~cell~~ exp. design for BLI formation.


TRANSPARENT WINDOW
TEFLON BEAKER
LEVEL OF BATHING MEDIUM
APERTURE
OUTER CHAMBER OF PLEXIGLAS
GLASS ENCAPSULATED MAGNETS
LAMP
MAGNETIC STIRRER
MICROSCOPE

sticking to teflon beaker. Then it was washed thoroughly in double distilled water and was soaked in ethanolic-HCl (.1M) for four hours to remove the trace of KOH remained on beaker during boiling. Again it was boiled in 100% Ethanol on mantle heater for one hour and soaked in fresh ethanol for about 4 hours.

The teflon beaker was placed on a plateform inside a plexiglas chamber and stuck in place by applying little amount of silicon greese at the botton of the beaker. One side of plexiglas chamber had a transparent window for viewing the BLM. The beaker and chambers were filled with an electrolyte (0.1 N NaCl) to the same level above the aperture of teflon beaker. For this we poured 75 ml 0.1N NaCl in chamber and 7.5 ml 0.1 N NaCl in teflon beaker.

The following method was used to check and adjust the levels of the electrolyte in the inner and outer chambers. The glass capillaries were dipped in the electrolyte, one inside the beaker and other in the outer chamber. Extra electrolyte was added to; or removed from either of the chambers until the liquid levels in the two capillaries were at the same height. The accurate adjustment of levels was very important as the difference in hydrostatic pressure causes bulging and loss in stability of BLM.

A little amount of phosphatedyl serine solution (in hexane, 1 mg/ml) was placed at the aperature using a fine brush. The lipid solution formed a thick lens at the aperture and gradually thinned. The thinning process was observed by focusing white light from a microscope lamp and the light reflected by lipid film was observed through a low powered travelling microscope. The lens like lipid

film gradually thins and colored interference fringes are seen. It took about 15 min to approach bilayer thickness which appears dull grey in color. We got 15 min stable BLM, after that it bursted.

B 2. Preparation of liposomes:

Egg lecithin solution (in chloroform: methanol 95: 5, provided by Mr. Sudipto Das) was taken in a test-tube and dried in N_2-atmosphere and weighed. The amount of 10mM Tris-HCl buffer (pH 7.2 with 0.02% Na azide) was so added that final concentration of lipid was 0.15 mg/ml. This suspension was vortexed for five min and then sonicated in ultrasonic disintegrator (MSE, England), for 30 min with 2 min steps cooling either in N_2- atmosphere or without N_2- atmosphere . The vial in which suspension was sonicated was dipped in ice water. ANS was added while preparing solution of lipid in 10mM Tris-HCl buffer (pH 7.2) making its final concentration 10^{-5}M. Sonicated suspension was centrifuged at around 28000 rpm in Vac-601 centrifuging machine for 90 min to remove the titanium particles from the sonication probe. Sonication was done at 8-10 μ amplitude. Supernatant of centrifuged suspension was taken as liposomes and used for fluorescence measurements.

B.3 Fluorescence measurements:

(i) Instrumentation: Fluorescence measurements were carried out using a fluorimeter designed and fabricated in our laboratory. Fig. 8 shows the schematic diagram of the instrument. A high pressure mercury lamp (HQE 40, West Germany) was used for the excitation. The excitation wave length was selected by a monochromator, M_1, (CSIO, Chandigarh, India) and focused on the sample

Figure 6:

Block diagram of spectrofluorometer used in this study.

(1) S, Mercury lamp (high pressure) HQS.40 Osram (W. Germany).

(2) C_1 C_2 C_3; Achromatic condensing lenses.

(3) M_1; Monochromator, CSIO (India) 300-800 nm.

(4) M_2; Monochromator, MDR-II, (U.S.S.R.).

(5) PM; Photomultiplier EMI 9550 QB, 520 response operated at 1200 V.

(6) Amplifier- Electrometer amplifier ECIL-Model EA-812.

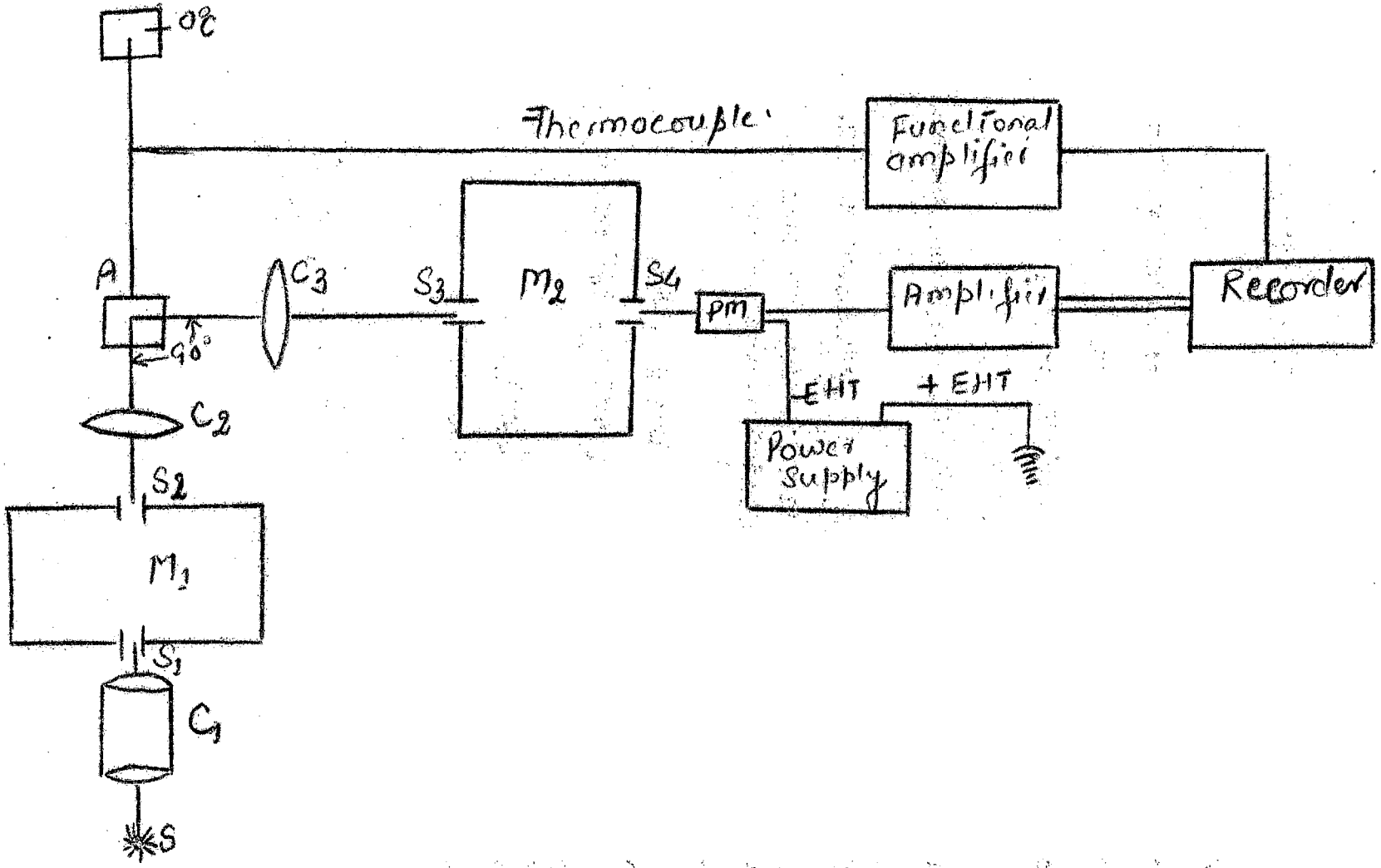
(7) Power supply; Regulated DC supply, +ive terminal is earthed and negative H.T. applied at the cathode of PM tube.

(8) Recorder; x-y Recorder, Rikken Denshi, F-43.

(9) S_1 S_2 S_3 S_4; adjustable slits S_1 and S_2; 0.5 nm, S_3 and S_4; 0.6 nm.

(10) A; Sample in 1 cm path length cuvette.

(11) Functional amplifier for thermocouple.



Block Diagram of the Spectrofloaromeler

with the help of schematic lenses. The fluorescence was observed in a direction perpendicular to the excitation beam. A second monochromator (MDR-II, U.S.S.R.) was used to scan the emission spectrum. The fluorescence light was detected by a photomultiplier tube (EMI 9558 QB) operated at -1200 V. The signal from photomultiplier was amplified and read by an electrometer amplifier (EA 812, ECIL, India). For the experiments the output of the amplifier was fed to x-y Recorder (F-43, Rikon, Denshi, Japan).

(ii) Measurements: The excitation wavelength was adjusted to 365 nm by M_1 monochromator. 10^{-5}M ANS in 10mM Tris-HCl buffer (pH 7.2 with 0.02% Na azide) was taken in cuvette and fluorescence spectra was measured, by scanning at different wavelengths on y axis of x-y recorder. Fluorescence spectra of liposomes was also measured in same manner.

(iii) Temperature induced phase transition: The cuvette containing the sample was kept in water jacketed cuvette holder and was heated by circulating water from a thermostatic water bath. A copper constantan thermocouple was used to measure temperature of sample and was connected to x-channel of the x-y recorder via an amplifier.

Three samples were measured for phase transition (1) ANS bound to liposomes prepared in N_2 atmosphere (2) ANS bound to liposomes prepared in Non-N_2 atmosphere (3) ANS bound to simply phosphatidyl cholines (Egg lecithin). Scan wavelength for fluorescence measurements in phase transition studies was adjusted at 490 nm.

RESULTS AND DISCUSSION

(1) Chloroplast membrane:

The effect of cooling and heating of sample on absorption characteristics have been studied by determining the difference spectra between two identical samples at different temperatures. The difference spectrum at each temperature is measured as

$$\Delta O.D_{(t)} = O.D_{(t)} - O.D_{(22^{\circ}C)}$$

Where t is any temperature at which the spectral changes are to be observed and 22°C is room temperature at which identical sample is kept throughout the experiment.

Upon cooling (22°C → 11.4°C), the difference spectrum of chloroplast suspension shows an increase in absorption at 410, 440, 470, 500 and 675 nm and a decrease in absorption at 690 nm(Fig 9a). The different spectrum curve, shown in Fig 9(a) at 12.5°C was measured while cooling the sample from 22°C to 11.4°C.

Upon heating the sample from 11.4°C to 29.5°C (11.4°C → 15.9°C → 19.9°C → 25°C → 29.5°C), a progressive decline in absorbance at 675 nm and an accompanying increase at 690 nm is observed (Fig 9a,b). It may be mentioned here that the existence of isobestic points at 660 nm (Fig 9a) and 690 nm (Fig 9b) is noticed. Heating the sample (11.4°C → 42.04°C) is accompanied by decreases in absorbance at 410, 440, 470 and 500 nm, except at 15.9°C and 19.9°C where absorption is more than that of at 11.4°C. This can be attributed to hysteresis effect. It is interesting to note that absorbance peaks have shifted to longer wavelengths

Figure 9:

Difference absorption spectrum as a function of temperature in cooling $\longrightarrow$ heating cycle.

(a) at 12.5°C, 11.4°C and 15.9°C.

Note: $O.D._{12.5°C}$ was measured during cooling (22°C $\longrightarrow$ 11.4°C)

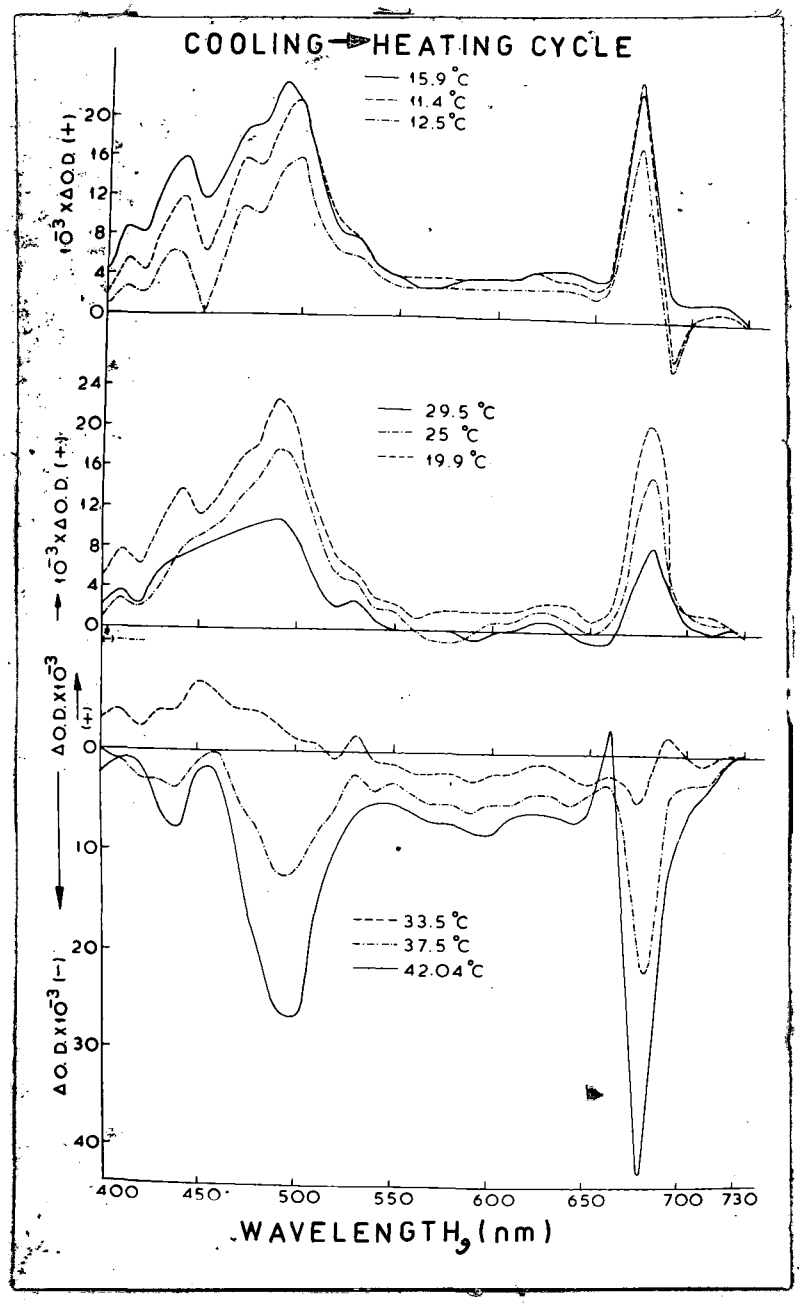
(b) at 19.9°C, 25°C and 29.5°C.

(c) at 33.5°C, 36.5°C and 42.0°C.

Difference spectrum is measured between two identical samples at different temperatures.

$$DD_{(t)} = O.D._{(t)} - O.D._{(22°C)}$$

t = temperature at which spectral change is observed.
22°C = room temperature.


COOLING → HEATING CYCLE
15.9 °C
11.4 °C
12.5 °C
10^{-3} x Δ O.D. (+)
(a)
29.5 °C
25 °C
19.9 °C
10^{-3} x Δ O.D. (+)
(b)
Δ O.D. x 10^{-3} (+)
Δ O.D. x 10^{-3} (−)
33.5 °C
37.5 °C
42.04 °C
(c)
400 450 500 550 600 650 700 730
WAVELENGTH (nm)

at 450, 480 and 680 nm for 440, 470 and 675 nm, respectively between 19.9°C and 29.9°C. At 32.5°C, absorption due to chlorophylls is similar to that of at 22°C and beyond 33.5°C, there are drastic decreases in absorbance at 440, 480, 500 and 680 nm (Fig 9c).

Reversibility of spectral changes at 410, 440, 470 and 475 nm as a function of temperature is observed during cooling → heating cycle but hysteresis effect is quite apparent in all cases.

Cooling the chloroplast (42.04 ⟶ 13.6°C) after heating cycle does not restore the original spectrum observed at corresponding temperature during cooling → heating cycle. During cooling the sample, a concomitant measurement of difference spectra at 36.4°C and 31.25°C shows that the absorbance was almost same as it was at 42.04°C but at 26.1°C absorbance increases abruptly at 420 and 660 nm (Fig 10). After stabilization the temperature at 26.1°C for about 5 min, further increase in absorbance is observed at 420 and 660 nm indicating the existence of hysteresis effect. Further cooling the sample (26.1 ⟶ 13.6°C) shows shifting of absorbance at 420 and 660 nm to 430 and 670 nm and appearence of another peak at 685 nm (Fig 11).

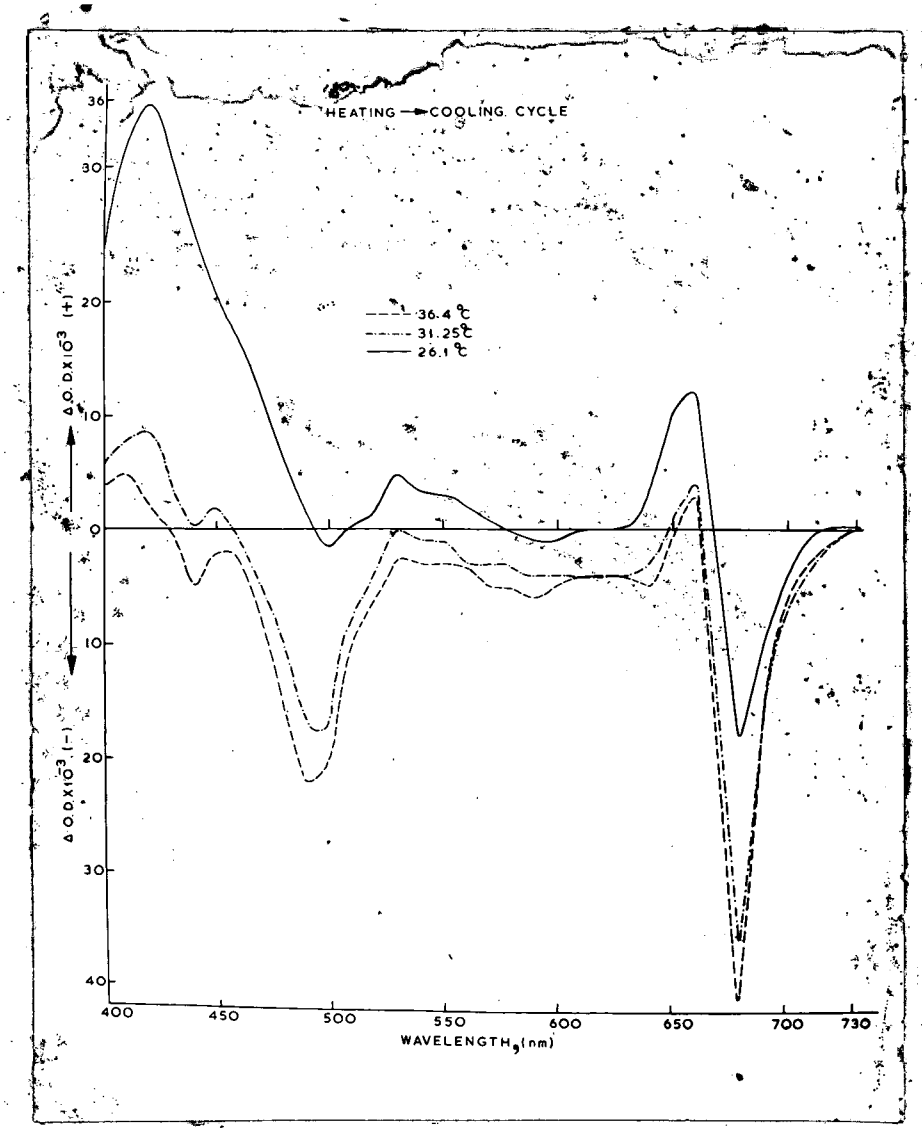
Hysteresis effect upon cooling and heating has been observed in studies of fluorescence, of chlorophyll in vivo (Murata, 1975), and of external fluorescent probe, ANS, in model membrane systems (Thauble and Overath, 1973). We, for the first time, observed the hysteresis effect in absorption studies of chlorophyll in chloroplast membrane. Heating of the sample under

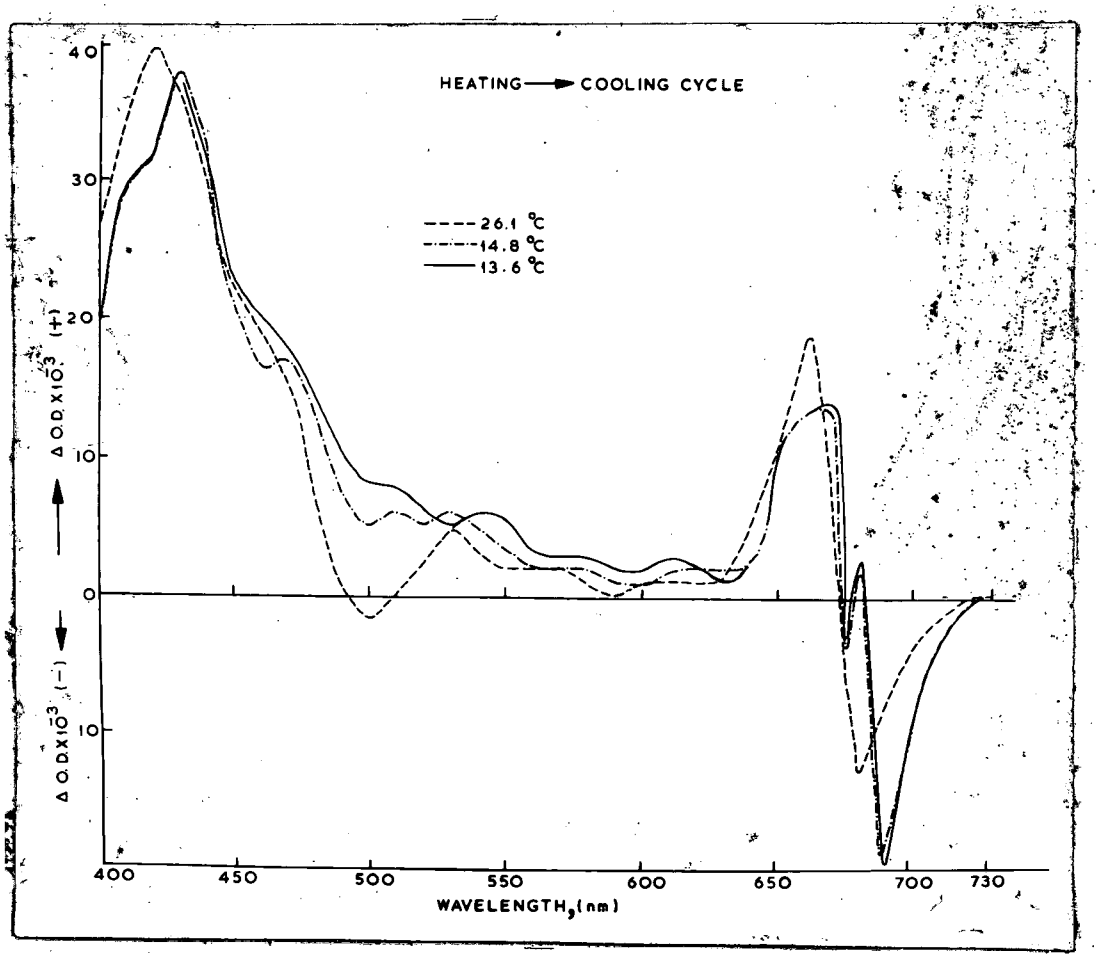
Figure 10:

Difference absorption spectra as a function of temperature in heating $\rightarrow$ cooling cycle; at 36.4°C, 31.25°C and 26.1°C. Difference spectra was measured in same way as indicated in legend of Fig 9.

Figure 11

Difference absorption spectra as a function of temperature in heating to cooling cycle at 26.1°C (after stabilization of temperature for 5 min), 14.8°C and 13.6°C.


HEATING → COOLING CYCLE
36.4 °C
31.25 °C
26.1 °C
Δ O.D X 10³ (+)
Δ O.D X 10³ (−)
WAVELENGTH, (nm)


HEATING → COOLING CYCLE
26.1 °C
14.8 °C
13.6 °C
Δ O.D X 10³ (+)
Δ O.D X 10³ (−)
WAVELENGTH, (nm)

aerobic conditions for a long time might irreversibly destory the pigments as well as lipid constituents of membrane due to their oxidation and degradation.

The lipids in chloroplast membrane undergo a phase transition upon raising the temperature above Tc (critical temperature), from solid to liquid crystalline state which is accessible to the chlorophylls (Murata, 1975) and thus they can form aggregates leading to increase in absorption at 690 nm. The pigments are bound to thylakoid membrane with some weak interactions, and increased temperature affects these interactions which may lead to increase in aggregate formation and it is quite possible that spectral changes may be associated with irreversible changes in aggregation.

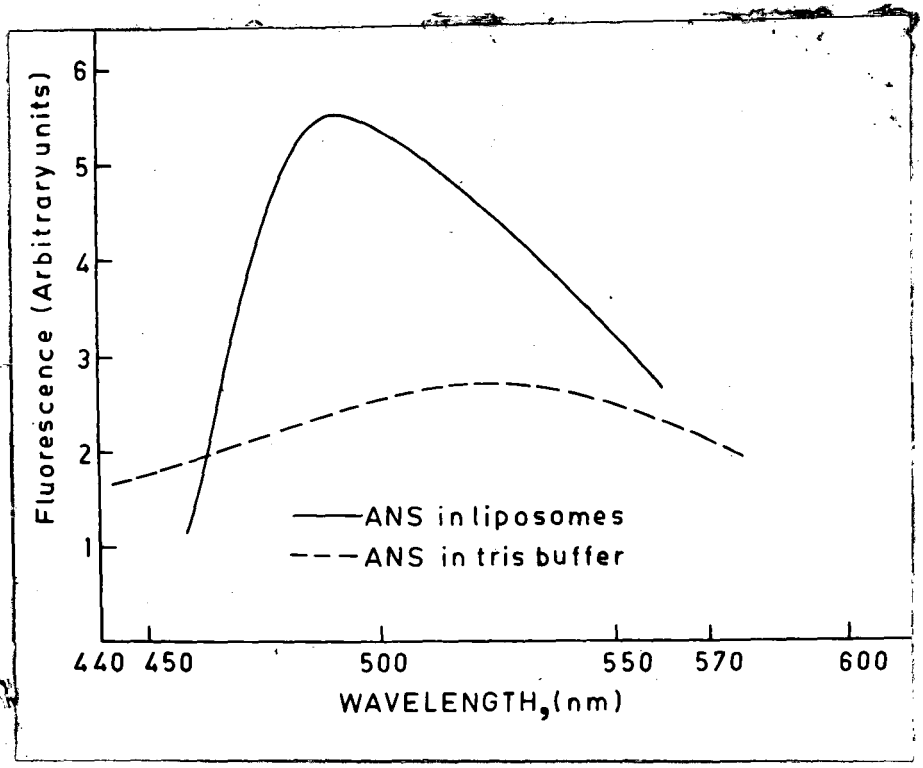
Beyond transition temperature loosening of membrane packing occurs and hydrophobic core of membrane may be exposed to water molecules which will destabilize the membrane conformation resulting into change in micro viscosity & polarity in the immediate environment of pigments and orientation of chromophore which may lead to change in absorption as well as shift in absorption peaks.

(2) Model membranes

The ANS fluoresces feebly at 523 nm in the buffer (10 mM Tris-HCl pH 7.2) but when bound to liposomes the fluorescence intensity increases sharply and a blue shift in fluorescence maximum is observed (Fig 12). The bound probe fluoresces at 490 nm.

Figure 12:

Emission spectra of ANS, in 10mM Tris-HCl buffer (pH 7.2, 0.02% Na-azide), ANS concentration 10^{-5}M; and bound to liposome (egg lecithin) suspension, ANS concentration 10^{-5}M, lecithin concentration 0.15 mg/ml; suspension buffer 10 mM Tris-HCl, pH 7.2 with 0.02% Na-azide. Excitation wavelength 365 nm.


Fluorescence (Arbitrary units)
6
5
4
3
2
1
ANS in liposomes
ANS in tris buffer
440
450
500
550
570
600
WAVELENGTH, (nm)

ANS is amphiphilic in nature having hydrophillic and hydrophobic regions and binds on semipolar surface of lipid structure to orient with hydrophilic group in the aqueous phase (Trauble, 1972). The fluorescence of ANS depends on Z value which is an index of polarity of solvent. Higher is Z value, higher is the polarity and therefore, lesser is fluorescence (Arindam, 1977, Prasad, 1976, Turner & Brand, 1968). This would explain the increase in ANS fluorescence bound to liposomes. When ANS is bound to liposomes, the decrease in polarity would increase fluorescence intensity and shift the fluorescence maximum towards lower wavelength.

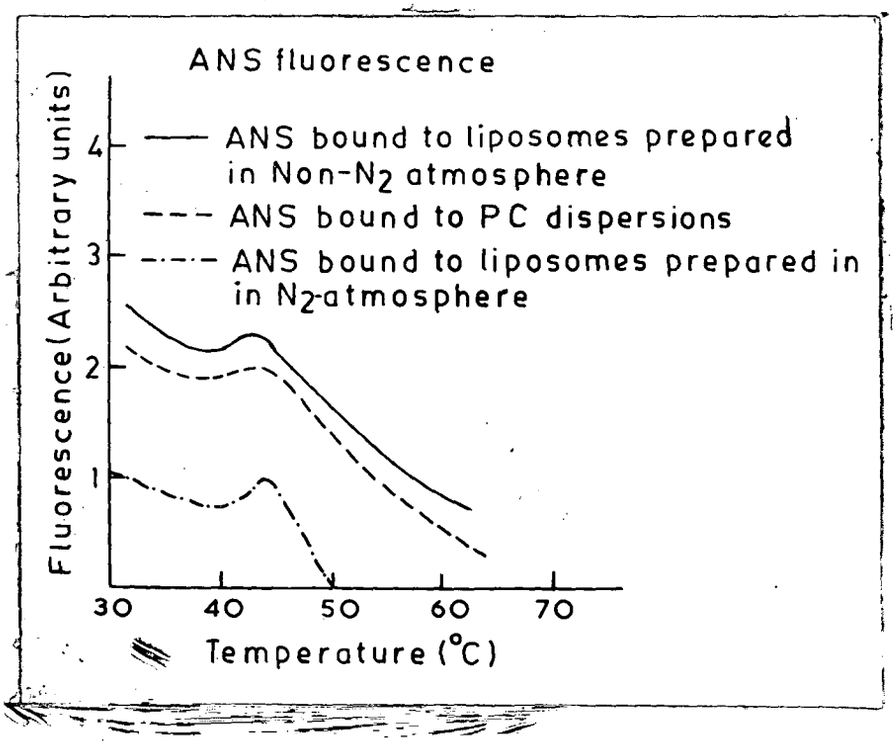
The blue shift in emission maximum of molecules has been described due to greater destabilization of polar solvents (Arindam, 1977) and position of electronic levels is also dependent on nature of solvent (Weber and Lawrence, 1954).

A phase transition at 44.5°C is quite sharp in liposomes prepared under N_2-atmosphere by showing a sharp increase in fluorescence of bound ANS (Fig 13). In case of ANS bound to PC dispersions and liposomes prepared under Non-N_2-atmosphere, the transition occurs at 43°C and 42.5°C respectively but sharpness is reduced in these preparations (Fig 13). In decrease in transition temperature of liposomes prepared in Non-N_2-atmosphere may be associated with the oxidation of lipids during sonication.

At phase transition, as lipid changes from solid to liquid crystalline state, there occurs loosening in the packing of the bilayer not only in hydrocarbon chain region but also in polar head group region to which ANS is bound (Trauble, 1972). It is

Figure 13:

Phase transition profile of liposomes and PC dispersions (egg lecithin). Concentration of egg lecithin 0.15 mg/ml, suspension buffer 10 mM Tris-HCl, pH 7.2 with 0.02% Na-azide, concentration of ANS, 10^{-5}M. Excitation wavelength 365 nm, scan wavelength 490 nm.


ANS fluorescence
ANS bound to liposomes prepared in Non-N2 atmosphere
ANS bound to PC dispersions
ANS bound to liposomes prepared in in N2-atmosphere
Fluorescence (Arbitrary units)
1
2
3
4
30
40
50
60
70
Temperature (°C)

suggested that gradual loosening precedes the main transition (solid ⟶ liquid crystalline) and increase in ANS fluorescence intensity at phase transition temperature is primarily due to increase in number of binding sites for ANS (Trauble, 1972).

The decrease in fluorescence intensity beyond the transition temperature may be the result of changes in polar head group mobility and changes in the packing of ANS between the polar head groups which ultimately causes the change in accessibility of water to ANS (Hayenes & Staerk, 1974) increasing polarity around the ANS. Vanderkooi and Mortonosi (1969) have also suggested the decrease in fluorescence of ANS bound to phospholipid micelles as a result of change in conformation of membrane, primarily in lipid bilayer.

BLM preparation is done and its stability is found for 15 min in our study.

R E F E R E N C E S

1. Arindam Sen (1977): Use of model membrane system for studies on lipid-protein interactions; Ph.D. thesis, Jawaharlal Nehru University, New Delhi-110067.

2. Bangham, A.D. (1978): Liposomes and their uses in Biology and medicine; Annals of New York Academy of Sciences, 18, 2.

3. Bangham, A.D., Hill, M.W. and Miller, N.G.A. (1974): Methods in membrane biology 1,1; Plenum Press, New York, N.Y.

4. Bangham, A.D., Horne, R.W., (1964): J. Mol. Biol. 8, 660.

5. Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965): J. Mol. Biol. 13, 238.

6. Batzri, S. and Korn, E.D. (1973): Biochim. Biophys. Acta, 298, 1015.

7. Burnner, J., Skrabal, P. and Hauser, H. (1976): Biochim. Biophys. Acta, 455, 322-331.

8. Colbow, C. (1973): Biochim. Biophys. Acta, 56, 791-796.

9. Davson, H. and Danielli, J.F. (1952): The permeability of natural membranes. 2nd edition, Cambridge University Press, p.57.

10. Galley, T.A. and Edelman, G.M. (1965): Biochim. Biophys. Acta, 94, 105.

11. Gorter, E. and Grendel, F. (1925): J. Exptl. Med. 41, 439.

12. Hayenes, D.H. and Staerk, H. (1974): J. Memb. Biol. 17, 313-340

13. Holton, R.W., Blecker, H.H., Ondonore, M.N. (1964): Photochem. 3, 595-602.

14. Huang, C. (1969): Biochemistry 8, 344-352.

15. Huang, C. and Thomson, T.E. (1965): J. Mol. Biol. 13, 183.

16. Huang, C. and Thomson, T.E. (1966): J. Mol. Biol. 15, 539.

17. Jain, M.K. (1972): The Bimolecular lipid membrane: A system, Van Nustrand Reinhold Co., New York.

18. Kruff, B.De, Demel, R.A., Slotboom, A.J., Van Deenen, L.L.M. and Rosenthol, A.F. (1973): Biochim. Biophys. Acta, 307, 1-19.

19. Lee, A.G. (1975): Biochemistry, 14, 2397-2401.

20. Lehninger, A.L. (1975): Biochemistry, p. 279, Kalyani Publishers Ludhiana, India.

21. Lenaz, G. (1977): Membrane proteins and their interactions with lipids, p. 47, Ed. Capaldi, Rhoderick A.; New York, Marcel Dekker.

22. Litman, B.J. and Thomson, T.E. (1967): Fed. Proc. 26, 834.

23. Mueller, P., Rudin, D.O., Tein, H.T. and Wescott, W.C. (1962): Nature, 194, 979.

24. Murata, N. (1975): Plant Physiology, 56, 791-796.

25. Nobel, Park S. (1976): Plant Cell Physiology, p. 13-19, W.H. Freeman and Company, San Francisco.

26. Prasad, Usha (1976): Studies of Structural and Functional Changes in Chloroplast Membranes, Ph.D. thesis, Jawaharlal Nehru University, New Delhi-110067, India.

27. Robertson, J.D. (1960): Prog. Biophys./Chem. 10, 343. Biophys.

28. Sandermann, H.J. (1978): Biochim. Biophys. Acta, 515, 209-237.

29. Singer, S.J., Nicholson, G.G. (1972): Science, 175, 720-731.

30. Small, D.M. (1970): Fed. Proc. 29(4): 1320.

31. Tein, H.T. (1970): Chlorophyll protein reaction centers and photosynthetic membrane, Brookhaven Symposia in Biology, No. 23, 104.

32. Trauble, H. (1972): Biomembranes, Vol. 3, 197. Plenum Press, New York, London.

33. Trauble, H. and Overath, P. (1973): Biochim. Biophys. Acta, 307, 491-512.

34. Turner, D.C. and Brand, L. (1968): Biochemistry, 7, 3381.

35. Vanderkooi, J. and Martonosi, A. (1969): Arch. Biochem. Biophys. 133, 153-163.

36. Weber, G. and Lawrence, D.J.R. (1954): Biochem. J., 56, 31.