BIOLOGICAL IMPLICATIONS OF LIQUID MEMBRANE PHENOMENA

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By
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Dedicated to my father
(Late) Shri O.P.Sharma,
who was a source of
inspiration to me.

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CERTIFICATE

This is to certify that the thesis entitled "BIOLOGICAL IMPLICATIONS OF LIQUID MEMBRANE PHENOMENA" submitted by Mr. Ravindra Kumar Sharma, ID No.80PM21002 for the award of the Ph.D. degree of the Institute, embodies original work done by him under my supervision.

Dated Aug 22, 1983.

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

Surface active molecules when added to aqueous phase in contact with a hydrophobic phase-say air-are known to accumulate at the air/water interface in such an orientation that the hydrophobic tails of surfactant molecules are preferentially directed towards air-the hydrophobic phase and the hydrophilic moities are in the aqueous phase. When concentration of the surfactant exceeds its critical micelle concentration (CMC) the surfactant molecules form aggregates which are known as micelles and stay in the bulk of the solution. It has also been known[1] that addition of soluble surfactants modifies material transport across interfaces between phases. Martin's discovery[2] that the addition of a small amounts,of the order of a few ppm,of surfactants like polyvinyl methyl ether (PVME) to saline feed in reverse-osmosis dramatically enhance the salt retention capacity of cellulose acetate membranes with but a small decrease in the flux of product water, was explained by Kesting's[3,4,5] on the basis of a liquid membrane hypothesis. According to this hypothesis the surfactant layer which forms spontaneously at the

cellulose acetate membrane/saline solution interface acts as a liquid membrane in the series with the supporting membrane and is responsible for the enhanced salt rejection. It was shown that as concentration of the surfactant is increased the interface becomes progressively covered with the surfactant layer liquid membrane and at the CMC it is completely covered.

Since molecules of the surface active nature are crucial to living matter and its organisation[6], the hypothesis that the surfactant layer generated at the interface acts as a liquid membrane can have significant biological implications. The present thesis contains an account of the investigations carried out with a view to exploring biological implications of "the liquid membrane phenomena". Attention has been focussed mainly on two aspects. These are : 1) workability of liquid membrane bilayers generated on a hydrophobic supporting membrane as model system for biomembranes and 2) role of liquid membranes generated by surface active drugs in the mechanism of their action.

Model Membrane System:

In a multicellular organism each cell communicates with its neighbouring cells in terms of mass transfer, energy transfer etc. If it is a unicellular organism it communicates with its environment. In any case the instruments of communication are embedded in the membrane enveloping the material contents of the cells. This is why biological membranes have come under intense investigations in recent years. However, owing to the complexity of the biological membranes, it has long been fashionable to experiment with artificially constituted membrane systems. This is done in the hope,and rightly so that investigations on these much similar model systems will provide insights for understanding similar and comparable phenomena in biological membranes. Biological membranes are visualised[7] as a matrix of lipid bilayers in a fluid, liquid-crystalline state with proteins incorporated in it. Since lipid bilayer is the backbone or the core of biological membranes several attempts have been directed to construct model system for this core. The bilayer lipid membrane or the black lipid membrane (BLM) as it

is also some times called, developed by Mueller, Rudin, Tien and Wescott[8-13] is the model system which has been most widely experimented with. Tien's excellent monograph[13] contains a comprehensive account of theoretical and practical aspects of BLM. It has been successfully demonstrated[12,13] that biologically relevant transport processes can be mimicked on BLM's after incorporating relevant proteins or other molecules in them. Although BLM, to date continues to be one of the most widely investigated model system, certain facts about BLM deserve a mention. The values of electrical resistance for BLM in general are very high[8,14-16] much higher than those reported for biomembranes[17]. Also the rate of passive ionic diffusion through BLMs is much slower[18,19] than through biomembranes[20,21]. This has been ascribed[22] to a tight molecular arrangement of lipid molecules in the BLMs while in biomembranes the lipid bilayer is in a fluid state[7].

The constituent lipids of biomembranes are surface active in nature and hence capable of generating liquid membranes at the interface

according to the liquid membrane hypothesis[3-5]. It should be possible, therefore, by suitable experimental manipulations, to generate from aqueous solutions of the lipids, one liquid membrane on either side of the hydrophobic supporting membrane. Can the liquid membrane bilayers thus generated work as model systems for biomembranes? Although a few investigations[23,24] have indicated in favour of such a possibility it is not enough. To assess this possibility it is desirable to mimic on the liquid membrane bilayers some such transport processes which have already been mimicked on BLMs. Attempts made in this direction are contained in Chapter 2.

Liquid Membranes in Drug Action:

A wide variety of drugs are known to be surface active in nature[25-31]. This does not appear to be a sheer chance because in a number of cases excellent correlations between surface activity and biological effects have been demonstrated[32-38]. While investigating the actions of drugs like reserpine, prenylamine, chlorpromazine, propranolol etc.

it has been concluded[39] that "irrespective of chemical structure the surface activity of psychotropic drugs mainly determines their potency to affect all kinds of membranes, especially that of catecholamine storing particles". Since structural requirements for surface activity are often similar to those for interaction of drugs with receptor sites[40] the correlations between surface activity and biological effects appear to indicate the possibility of a common mode of action for surface active drugs. In view of the liquid membrane hypothesis it is logical to suspect that the liquid membranes generated by the surface active drugs at the site of their action acting as a barrier to the transport of relevant permeants might be an important step common to the mechanism of all surface active drugs. The investigations carried out recently on wide variety of drugs namely haloperidol[41], reserpine[42], chlorpromazine[43], imipramine[44] and anti-histamines[45] have strongly substantiated this surmise. Nevertheless, there is a need to investigate many more drugs belonging to different pharmacological categories for the role of the liquid

membranes generated by them in the mechanism of their action. Studies conducted on diazepam, local anaesthetics, propranolol and steroidal drugs, with this object in view, are contained in Chapter 4. This is preceded by Chapter 3 containing a review on the surface activity of drugs. It is collection of such literature reports which indicate that surface activity might play a significant role in the mechanism of action of the drugs.

The liquid membrane generated by the drug, itself, acting as a barrier modifying access of relevant permeants to the receptors is a new facet of drug action which has hitherto gone unnoticed. The reason for this appears to be the passive nature of transport which has traditionally been considered unimportant for biological action. The investigations contained in Chapter 4 highlight the role of passive transport through the liquid membranes generated by surface active drugs in the mechanism of their action.

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CHAPTER 2

PHOTO-OSMOSIS THROUGH LIQUID MEMBRANE BILAYERS

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PHOTO-OSMOSIS THROUGH LIQUID MEMBRANE BILAYERS

Black lipid membranes (BLMs) have been sucoessfully used as model systems for biological membranes[1]. Nevertheless, as stated in Chapter 1, certain facts about BLMs cannot be lost sight of. The lipid molecules are tightly packed within BLMs[2] while the lipid bilayer in biomembranes is in a highly fluid state[3]. In addition BLMs invariably contain in them some residual solvent. All this is menifested in the values of electrical resistances and of the ionic diffusion through BLMs. The values of electrical resistances for BLMs are several order of magnitude higher[4-8] than those for biomembranes[9] and also rate of ionic diffusion through BLMs is much slower[10,11] than through biomembranes[12,13].

In a recent study[14,15] bilayers of liquid membrane have been generated on a hydrophobic supporting membrane using Kesting's hypothesis, from lecithin, cholesterol and lecithin-cholesterol mixtures. The agreement, though qualitative, of the transport data

for the lecithin-cholesterol liquid membrane bilayer with those for the living membrane/BLMs was encouraging and indicated that the liquid membrane bilayers thus generated could also work as model systems for bio-membranes. To assess this possibility it is desirable to simulate some of the biologically relevant transport processes on such liquid membrane bilayers. Light induced transport processes through thylakoid membrane of the chloroplast afford an example where such a simulation can be attempted. Light gradients across chloroplast BLMs have been shown[16] to induce volume flux. The light induced volume flux, which can be termed as "photo-osmosis", is considered to be a consequence of electrical potentials developed across the BLMs due to action of light - the photo-electric effect[16].

Chloroplast extract is known[17] to be surface active in nature and hence according to Kesting's hypothesis[18] can generate a surfactant layer liquid membrane which would cover the interface completely at a concentration equal to or greater than its critical micelle concentration (CMC). Therefore, it should be possible using the procedure employed

in earlier studies[14,15] to generate liquid membranes from chloroplast extract on either side of a hydrophobic supporting membrane. The chloroplast liquid membrane bilayers thus generated should also show the phenomenon of photo-osmosis. Not only should they show the phenomenon, the trends in the data on photo-osmotic transport should also be consistent with the trends reported in chloroplast BLMs. Experiments undertaken with a view to demonstrating this are described and discussed in this Chapter. Haemoglobin which is known[19] to be surface active and also photo-conducting[20,21] and has structural similarity with chlorophyll - both chlorophyll and haemoglobin have porphyrin ring - has also been experimented with. Cyanocobalamin (Vitamin B_{12}) whose central structure - 'Corrin' ring system - is very similar to that of the porphyrins has also been investigated for the phenomenon of photo-osmosis. As we will see in the subsequent sections all the three substances viz. chloroplast extract, haemoglobin and cyanocobalamin showed the phenomenon of photo-osmosis with trends in the data, consistent with the trends reported in chloroplast - BLMs.

Materials and Methods:

Chloroplast extract was obtained from spinach leaves using the method described in literature[22]. The flow chart of the method is given in Fig. 1. The final fraction was evaporated to dryness and the residue was dissolved in ethanol to make stock solution of known concentration. Aqueous solutions of chloroplast extract of desired concentrations were prepared by adding known volume of ethanolic stock solution to aqueous phase with constant stirring. The amount of ethanol in the final solution was not allowed to exceed 0.1% by volume because it was shown by a control experiment that 0.1% solution of ethanol does not lower the surface tension of water to any measurable extent. Haemoglobin (human type IV) and cyanocobalamin used in these experiments were all obtained from Sigma (Cat.No.H7379 and V2876 respectively). All other chemicals used in the present experiments were of Analar grade. Distilled water distilled once in an all pyrex glass still was used for preparing solutions. The pH of all solutions was maintained 5 using a 0.1M acetate buffer. The critical micelle concentrations (CMC) of aqueous chloroplast extract, aqueous haemoglobin and aqueous

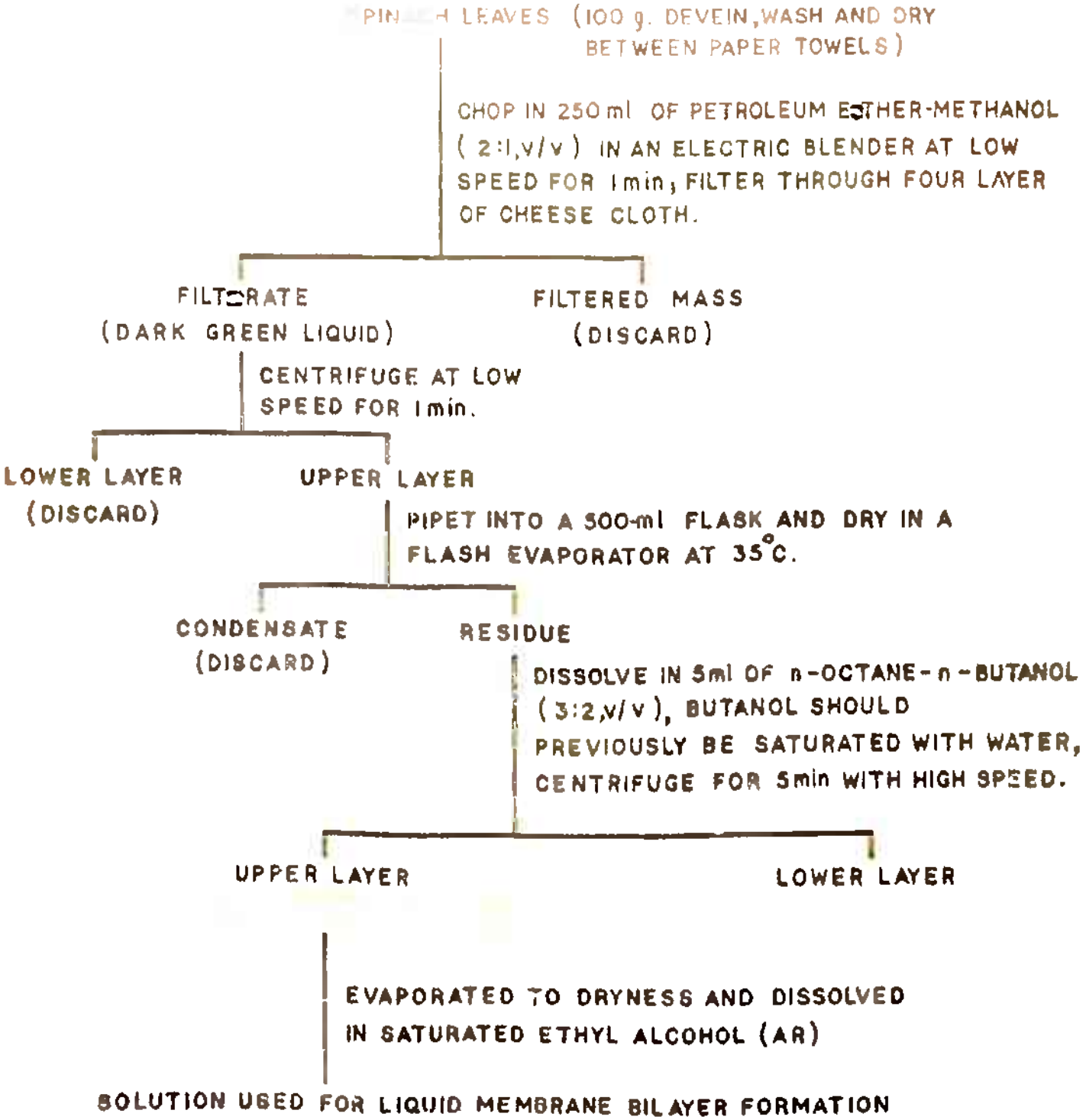

Fig. 1. Flow chart showing the method for the extraction of Chloroplast extract (Taken from Ref. 22).

cyanocobalamin as determined from the variation of surface tension with concentration were found to be 23.2 ppm, 12.0 ppm and 2.0 ppm respectively. The surface tensions were measured using a Fisher Tensiomat Model 21. The all glass transport cell used for hydraulic permeability measurements and photo-osmotic velocity measurements is described in Fig. 2. A Sartorius cellulose acetate microfiltration membrane (Cat.No. 11107) of thickness 1×10^{-4}m and area $2.55 \times 10^{-5} m^2$ which acted as a support for the liquid membranes separated the transport cell in to two compartments C and D. During hydraulic permeability measurements the entire cell except the capillary (Fig. 2) was covered with black paper to protect it from exposure to light and the electrodes E_1 and E_2 were short circuited to eliminate the possiblity of electro-osmotic back flow, due to streaming potential developed across the membrane, being a disturbing factor in the hydraulic permeability measurements. For obtaining the hydraulic permeability data, which was exploited to demonstrate the existence of liquid membrane, the compartment C of the transport cell was filled with aqueous solutions of varying concentrations of the photo-active materials and the

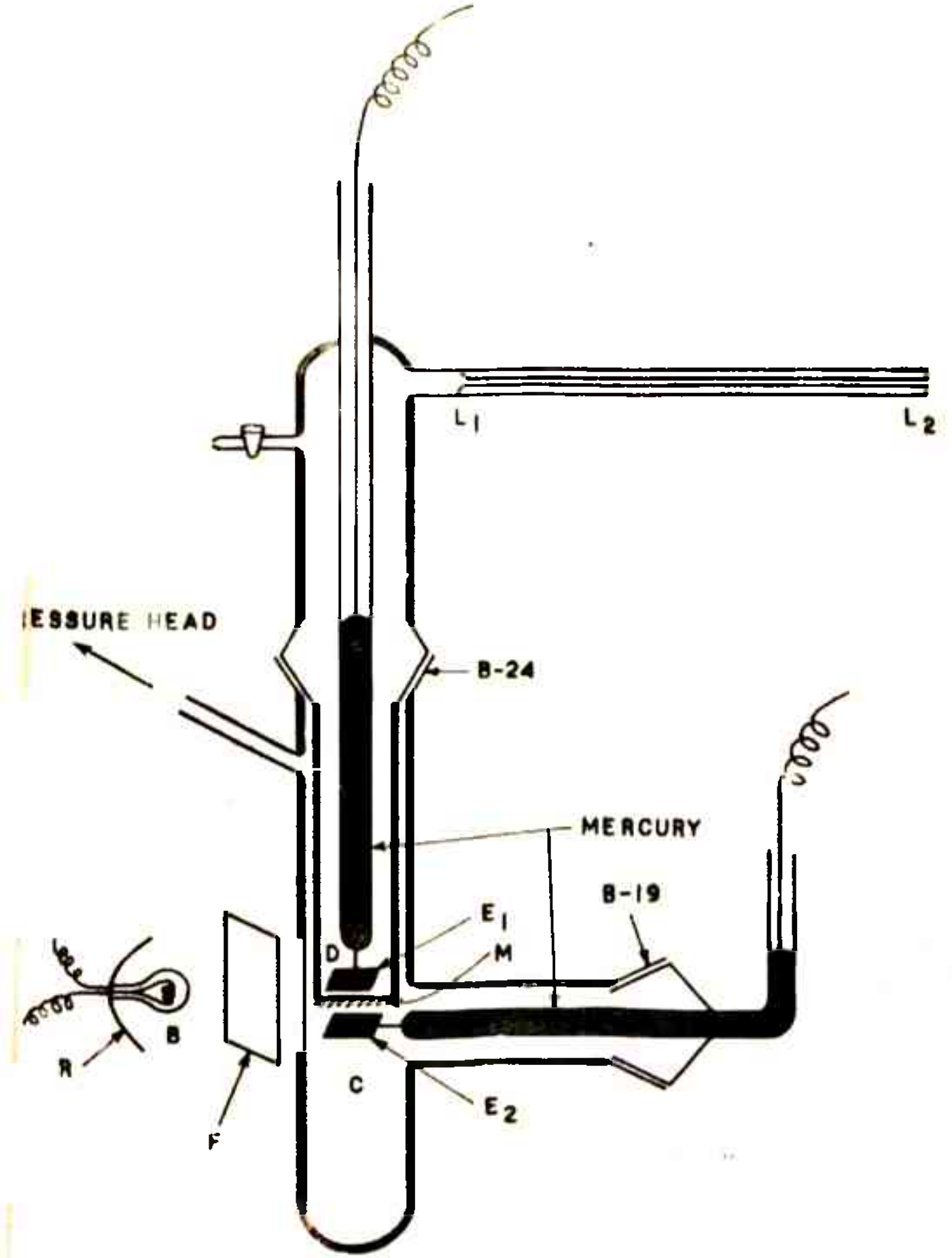

Fig.2. The transport cell. The thick lines indicate the blackened portions: R - reflector; B - 100 W bulb; F- optical filter; E_1 and E_2 platinum electrodes; M - the supporting membrane. The concentrations of chloroplast extract, haemoglobin, protoporphyrin and cyanocobalamin used in photo-osmosis experiments were 46.4 ppm, 25.8 ppm, 1.5 ppm and 4.0 ppm respectively.

compartment D was filled with water. Known pressures were applied on the compartment C by adjusting the pressure head (Fig. 2) and the volume flow in the capillary L_1 L_2 was noted using a cathetometer reading upto 0.001 cm and a stop watch reading upto 0.1 sec.

For measurements of photo-osmotic velocity the experimental set up is also described in Fig. 2. The compartment C (Fig. 2) was filled with aqueous solutions containing desired concentrations of the photo-active materials and electron acceptors and the compartment D was filled with the aqueous solutions containing the same concentration of the photo-active materials and desired concentrations of electron donors. The concentration of photo-active materials was higher than their CMC. The condition of no net pressure difference, $\Delta P = 0$, was imposed on the system by adjusting the pressure head. The light was then switched on and the consequent movement of liquid meniscus in the capillary L_1 L_2 was noted with time. During the measurements of photo-osmotic velocity, a constant and stabilized voltage at 220 volts from A.C. mains was fed to the bulb B (Fig. 2) and the distance between the transport cell and the bulb was kept fixed.

In order to study the variation of photo-osmotic velocity with intensity of the incident light various voltages were fed to the bulb to alter the intensity of the light and the consequent volume fluxes were noted in the capillary L_1 L_2. To measure photo-osmotic velocity in presence of externally applied electric field the electrodes E_1 and E_2 were connected to an electronically operated stabilized D.C. power supply (Systronics, Type 612). The electrode E_2 was connected to the positive terminal of the power supply to make the lower compartment C positive with respect to the dark compartment. The pressure head was suitably adjusted to balance the volume flux induced by externally applied voltage in the capillary L_1 L_2. When the liquid meniscus in the capillary became stationary the light was allowed to fall on the membrane in the lower compartment and the consequent volume flux in the capillary L_1L_2 was noted. This was repeated at several values of externally applied voltages.

All experiments were done at constant temperature using a thermostat set at $40 \pm 0.1^{\circ}C$.

Results and Discussion:

The hydraulic permeability data in presence of various concentrations of all the three photo-active materials viz. chloroplast extract, haemoglobin and cyanocobalamin were found to be in accordance with the proportional relationship.

$$Jv = L_p \Delta P \qquad (1)$$

Where Jv is the volume flux per unit area of the membrane, ΔP is the applied pressure difference and L_p is the hydraulic conductivity coefficient. The values of L_p obtained from the slopes of such plots show a progressive decrease with increase in the concentrations of the photo-active materials upto the CMC, beyond which they become more or less constant. The normalised values of hydraulic conductivity coefficients i.e. the values of L_p/L_p^o where L_p^o is the value of L_p when no photo-active material was used are plotted in Fig. 3. The trend in Fig. 3 is in keeping with Kesting's hypothesis[18] according to which as concentrations of the surfactant is increased the supporting membrane gets progressively covered with the surfactant layer

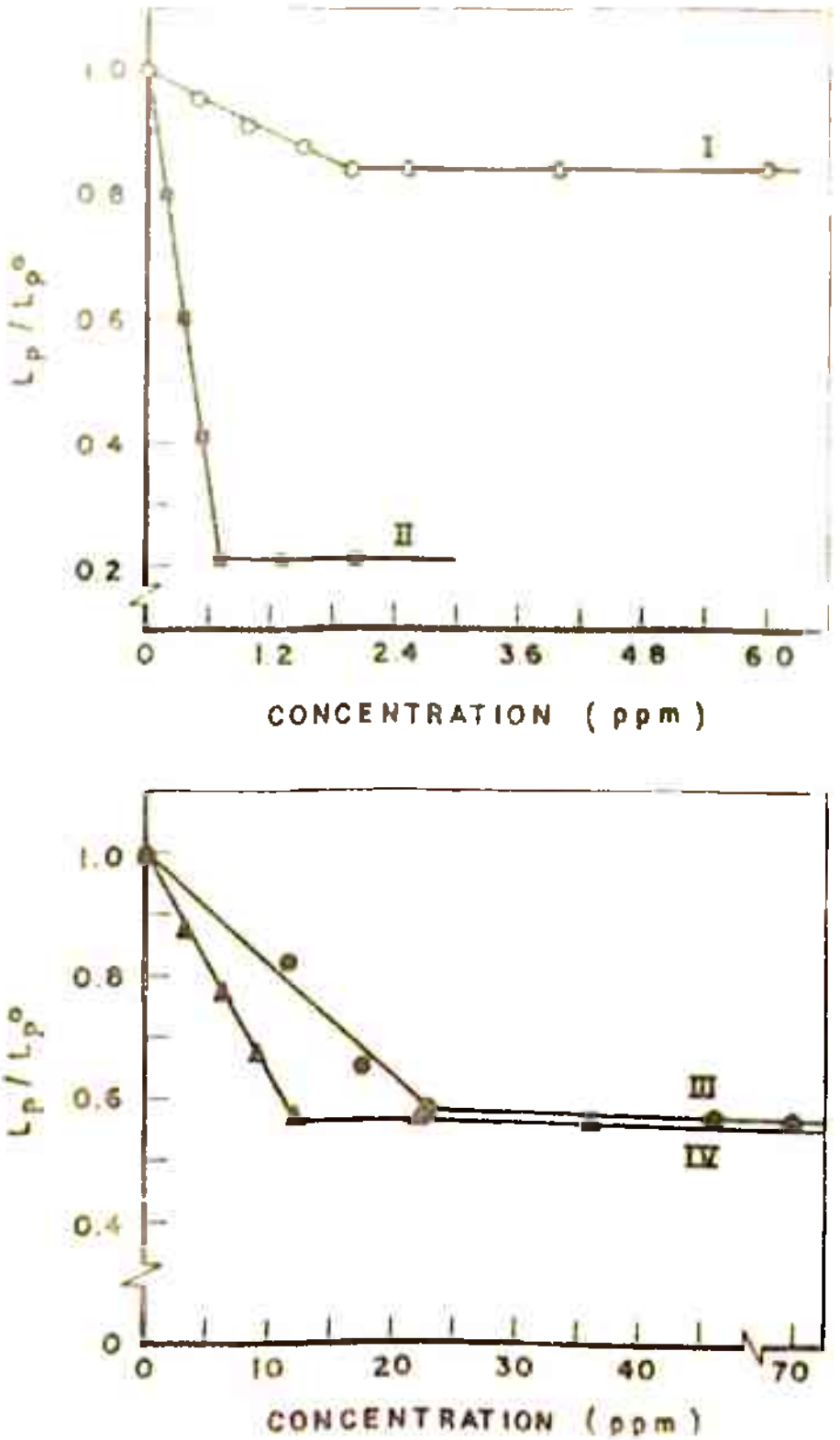

Fig. 3. Variation of L_p/L_p^o with concentration of photo-active materials. Curve I is for Cyanocobalamin, curve II is for Protoporphyrin, curve III is for Haemoglobin and curve IV is for Chloroplast extract.

liquid membrane and at the CMC it is completely covered. Analysis of the data on Lp in light of the mosaic membrane model[23-25] further confirms the existence of the liquid membrane in series with the supporting membrane. Since, according to the liquid membrane hypothesis[18] at CMC, the supporting membrane is fully covered with the surfactant layer liquid membrane, at concentrations lower than the CMC, it will be only partially covered. The situation is pictorially depicted in Fig. 4. The equation for volume flux for such a situation can be written as

$$Jv\ (A^s + A^c) = J_v^s\ A^s + J_v^c\ A^c \qquad (2)$$

where A represents the area of the membrane denoted by the superscripts and the superscripts s and c represents the bare supporting membrane and the supporting membrane covered with the liquid membrane respectively. In view of the linear relationship between Jv and ΔP, i.e. equation (1) and equation (2) can be transformed into

$$Jv = \left[L_p^s \{A^s/(A^s + A^c)\} + L_p^c \{A^c/(A^c + A^s)\}\right] \Delta P \qquad (3)$$

Fig. 4. The schematic representation of mosaic membrane formed when the concentration of the surfactant is lower than its critical micelle concentration. J_v^s, J_v^c, A^s and A^c have the same meaning as in equation (2).

Functionally L_p^s and L_p^c represents the value of L_p at 0 and at CMC respectively. The concept of progressive coverage in the liquid membrane hypothesis implies that at half the CMC, the fraction of the total area of the supporting membrane covered with the liquid membrane will be half and hence the slope of Jv vs. ΔP plot, in view of the equation (3) should be equal to $(L_p^s + L_p^c)/2$. Similarly, when concentration of the surface active agent is one fourth its CMC, the value of the slope should be equal to $\left[(3/4)\ L_p^s + (1/4)\ L_p^c\right]$ and so on. Thus in general terms if concentration of the surfactant is n times its CMC, n being less than or equal to 1, the value of the slope of Jv vs. ΔP plots should be equal to $\left[(1 - n)\ L_p^s + n\ L_p^c\right]$. The values of L_p thus computed at concentrations below the CMC in case of chloroplast extract match favourably with the experimentally determined values (Table I). Similar trends were found in the data for haemoglobin and cyanocobalamin lending support to the formation of liquid membrane in series with the supporting membrane. It is expected that in the liquid membranes. thus generated hydrophobic ends of the surface active materials will be preferentially oriented towards

Table I Values of L_p at various concentrations of Chloroplast Extract.

Concentrations (ppm)	0.0	11.592 (0.5CMC)	17.388 (0.75CMC)	23.184 (CMC)	46.368	69.552
L_p^{*} x10^8($m^3 s^{-1} N^{-1}$)	2.611	2.185	1.697	1.513	1.480	1.450
	±0.114	±0.133	±0.083	±0.046	± 0.036	±0.053
L_p^{**} x10^8($m^3 s^{-1} N^{-1}$)	-	2.062	1.787			
		±0.080	±0.063			

* Experimental values.

** Calculated values using mosaic model.

the hydrophobic supporting membrane. Now if the two compartments of the transport cell (compartments C and D in Fig. 2) are each filled with the solutions of the surface active material - chloroplast extract or haemoglobin or cyanocobalamin, of concentration higher than their CMCs, the supporting membrane would be sandwiched between the two layers of the liquid membrane generated on either side of it.

The data on photo-osmotic volume flux through the liquid membrane bilayers thus generated from chloroplast extract, haemoglobin and cyanocobalamin are recorded in Table II to VI and in Fig. 5. The lighted compartment always contained an electron acceptor and the dark compartment an electron donor. A general observation in these experiments was that the direction of photo-osmotic flow was always from the lighted compartment to the dark compartment.

Tien's observations on light induced water flow across chloroplast - BLMs were explained[16,26,27] in terms of semiconductor physics and classical electrokinetics. When a beam of light excites the BLM, electrons and holes are produced. Since electrons and holes have different life times and

mobilities, a separation of charges in the BLM results, leading eventually to a potential difference across the membrane, The light induced voltage across the BLM was considered to be the primary driving force for photo-osmosis. Similar explanation can be extended in the present case also to account for the originof the effect and direction of the flow. The chloroplast liquid membrane on excitation by light ejects electrons which are captured by the electron acceptors e.g. Fe^{+++} ions present in the illuminated compartment. Upon reduction of Fe^{+++} ions by photo-electrons or hydrated electrons an electrical double layer is generated which consists of a layer of anions in the solution - the mobile phase of the double layer and a layer of positively charged oxidised chloro-plast in the membrane phase. Since the illuminated compartment where electrons are generated due to the action of light is negative with respect to the dark compartment the negatively charged mobile phase of the double layer moves from the illuminated compart-ment to the dark compartment. Similar explanation can be offered in the case of haemoglobin and cyano-cobalamin.

Although the present experiments were carried out under constant temperature conditions, the possibility of the thermal gradients produced by light absorbtion, causing the observed flow has to be ruled out. The observations that, in the present experiments, as soon as the light was switched on, movement of the liquid meniscus in the capillary L_1 L_2 was noticed instantaneously and also as soon as light was switched off the flow stopped instantaneously strongly suggest that the observed volume flux can not be on account of thermal gradients because establishment and abolition of thermal gradients can not be an instantaneous process. It was also observed that on short-circuiting the electrodes E_1 and E_2 the light induced volume flux stopped completely. This observation not only rules out the possiblity of the thermal gradients being a cause for the observed flow but also confirms that the light induced voltage across the membrane is the primary cause for the observed photo-osmosis as suggested by Tien in the case of chloroplast BLMs[16].

Magnitudes of the electrical potentials developed across the pigmented BLMs, when it is illuminated

from one side, are known[26] to be enhanced many-fold in asymmetrical systems e.g. when different redox chemicals are present in the two bathing solutions separated by the pigmented BLM. Since the light induced voltage difference is the primary driving force for the observed photo-osmotic flux, the magnitude of the photo-osmotic velocity should vary with the choice of the redox chemicals in the two compartments of the transport cell. To study this, two sets of experiments were performed. In the first set of experiments Fe^{++} ions of concentration 1×10^{-3}M were kept in the dark compartment and two different electron acceptors of concentration 1×10^{-3}M, namely, ferric chloride ($FeCl_3$) and sodium sulphide (Na_2S) were taken in the illuminated compartment. The data in Table II reveal that the magnitude of the photo-osmotic velocity in all the cases viz. chloroplast extract, haemoglobin and cyanocobalamin when Na_2S was in the illuminated compartment is greater than the magnitude when ferric chloride was taken instead. This is consistent with the fact that Na_2S is stronger electron acceptor than ferric chloride[26]. Similarly in the second set of experiments ferric chloride of concentration 1×10^{-3}M

Table II Values of photo-osmotic velocity using different electron acceptors in the illuminated compartment.

	Electron acceptor in the illuminated compartment		Photo-osmotic velocity $Jv \times 10^5 (m.sec^{-1})$
Chloroplast Extract	$FeCl_3$	($1x10^{-3}M$)	0.358 ± 0.027
	Na_2S	($1x10^{-3}M$)	0.419 ± 0.028
Haemoglobin	$FeCl_3$	($1x10^{-3}M$)	0.248 ± 0.009
	Na_2S	($1x10^{-3}M$)	0.427 ± 0.004
Protoporphyrin	$FeCl_3$	($1x10^{-3}M$)	1.408 ± 0.055
	Na_2S	($1x10^{-3}M$)	1.945 ± 0.101
Cyanocobalamin	$FeCl_3$	($1x10^{-3}M$)	0.817 ± 0.012
	Na_2S	($1x10^{-3}M$)	0.853 ± 0.018

The dark compartment in all cases contained Fe^{++} ions ($1 \times 10^{-3}M$).

Table III Values of photo-osmotic velocity using different electron donors in the dark compartment.

	Electron donors in the dark compartment		Photo-osmotic velocity $Jv \times 10^5 (m.sec^{-1})$
Chloroplast Extract	NaI	($1x10^{-3}M$)	0.615 ± 0.001
	$K_4Fe(CN)_6$	($1x10^{-3}M$)	0.497 ± 0.010
	$Na_2S_2O_3$	($1x10^{-3}M$)	0.450 ± 0.003
	$FeSO_4(NH_4)_2SO_4$	($1x10^{-3}M$)	0.358 ± 0.027
Haemoglobin	NaI	($1x10^{-3}M$)	0.381 ± 0.019
	$K_4Fe(CN)_6$	($1x10^{-3}M$)	0.330 ± 0.001
	$Na_2S_2O_3$	($1x10^{-3}M$)	0.293 ± 0.013
	$FeSO_4(NH_4)_2SO_4$	($1x10^{-3}M$)	0.248 ± 0.009
Protoporphyrin	NaI	($1x10^{-3}M$)	2.699 ± 0.088
	$K_4Fe(CN)_6$	($1x10^{-3}M$)	2.293 ± 0.084
	$Na_2S_2O_3$	($1x10^{-3}M$)	1.982 ± 0.151
	$FeSO_4(NH_4)_2SO_4$	($1x10^{-3}M$)	1.408 ± 0.055
Cyanocobalamin	NaI	($1x10^{-3}M$)	1.256 ± 0.030
	$K_4Fe(CN)_6$	($1x10^{-3}M$)	0.964 ± 0.030
	$Na_2S_2O_3$	($1x10^{-3}M$)	0.913 ± 0.020
	$FeSO_4(NH_4)_2SO_4$	($1x10^{-3}M$)	0.817 ± 0.012

The illuminated compartment in all cases obtained Fe^{+++} ions ($1 \times 10^{-3}M$).

photo-osmotic effect, the values of photo-osmotic velocity should increase with the increasing concentrations of electron acceptors. The data on photo-osmotic velocity in all the three cases at various concentrations of Fe^{+++} ions in the illuminated compartment keeping the concentration of Fe^{++} ions in the dark compartment constant (1×10^{-3}M) confirms such a trend (Table IV). As a corollary of this it should be expected that if an external electric field is applied across the liquid membrane bilayers making the illuminated compartment positive with respect to the dark compartment the magnitude of the photo-osmotic velocity should increase with increase in applied voltage across the membrane. The data on the variation of photo-osmotic velocity with externally applied voltage were obtained in two cases - one in which Fe^{+++} ions (1×10^{-3}M) were present in the illuminated compartment and Fe^{++} ions (1×10^{-3}M) in the dark compartment and the other in the absence of electron donor and acceptor species i.e. Fe^{+++} and Fe^{++} ions in either compartment. The data in both the cases (Table V) for chloroplast liquid membrane bilayer confirms the expectation. The data on haemoglobin

Table IV Values of photo-osmotic velocity at various concentrations of electron acceptor (Fe^{+++} ions) in the illuminated compartment.

	Concentration of Fe^{+++} ions in the illuminated compartment	Photo-osmotic velocity Jv x 10^5 (m. sec^{-1})
Chloroplast Extract	1 x 10^{-4}M	0.096 ± 0.020
	5 x 10^{-4}M	0.186 ± 0.056
	1 x 10^{-3}M	0.358 ± 0.027
	5 x 10^{-3}M	0.598 ± 0.041
	1 x 10^{-2}M	0.756 ± 0.013
Haemoglobin	1 x 10^{-4}M	0.071 ± 0.003
	5 x 10^{-4}M	0.172 ± 0.004
	1 x 10^{-3}M	0.248 ± 0.009
	5 x 10^{-3}M	0.406 ± 0.017
	1 x 10^{-2}M	1.308 ± 0.016
Protoporphyrin	1 x 10^{-4}M	0.731 ± 0.047
	5 x 10^{-4}M	1.301 ± 0.110
	1 x 10^{-3}M	1.408 ± 0.055
	5 x 10^{-3}M	2.101 ± 0.188
	1 x 10^{-2}M	2.422 ± 0.128

contd....

Table IV contd.

	Concentration of Fe^{+++} ions in the illuminated compartment	Photo-osmotic velocity Jv x 10^5 (m. sec^{-1})
Cyanocobalamin	1 x 10^{-4}M	0.496 ± 0.038
	5 x 10^{-4}M	0.567 ± 0.016
	1 x 10^{-3}M	0.817 ± 0.012
	5 x 10^{-3}M	1.714 ± 0.089
	1 x 10^{-2}M	2.055 ± 0.094

The dark compartment in all cases contained Fe^{++} ions (1 x 10^{-3}M).

Table V Values of photo-osmotic velocity through chloroplast liquid membrane bilayers at different externally applied voltages.

	External applied voltage (volts)	Photo-osmotic velocity $Jv \times 10^{5}$ (m. sec^{-1})
(a) When the dark compartment contained Fe^{++} ions (1×10^{-3}M) and the illuminated compartment contained Fe^{+++} ions (1×10^{-3}M).	0.1	0.121 ± 0.003
	0.2	0.236 ± 0.022
	0.3	0.317 ± 0.011
	0.4	0.403 ± 0.013
	0.5	0.485 ± 0.002
(b) In the absence of Fe^{++} ions or Fe^{+++} ions in either compartment.	1.0	0.661 ± 0.003
	1.1	0.829 ± 0.005
	1.2	1.044 ± 0.010
	1.3	1.246 ± 0.015
	1.4	1.358 ± 0.001
	1.5	1.509 ± 0.002

and cyanocobalamin liquid membrane bilayer could not be obtained because the application of even small voltages caused electrolysis.

The open circuit photo voltages (E_{op}) in the case of chloroplast - BLMs are known to be dependent on the intensity of exciting light (I). The dependence has been found to be given by the following equation[36]

$$E_{op} = \ell \log (1 + I/L) \qquad (4)$$

where ℓ and L are constant for a given chloroplast BLM at a particular temperature. Under conditions of low light intensities, E_{op} becomes directly proportional to I as has indeed been found to be the case. As an implication of this it follows that the photo-osmotic velocity through the liquid membrane bilayers should also show a similar dependence on the intensity of exciting light. The data in Fig. 5, in the case of chloroplast extract and haemoglobin and cyanocobalamin, indeed show such a dependence on the intensity of exciting light.

The values of photo-osmotic velocity, for chloroplast extract, haemoglobin and cyanocobalamin,

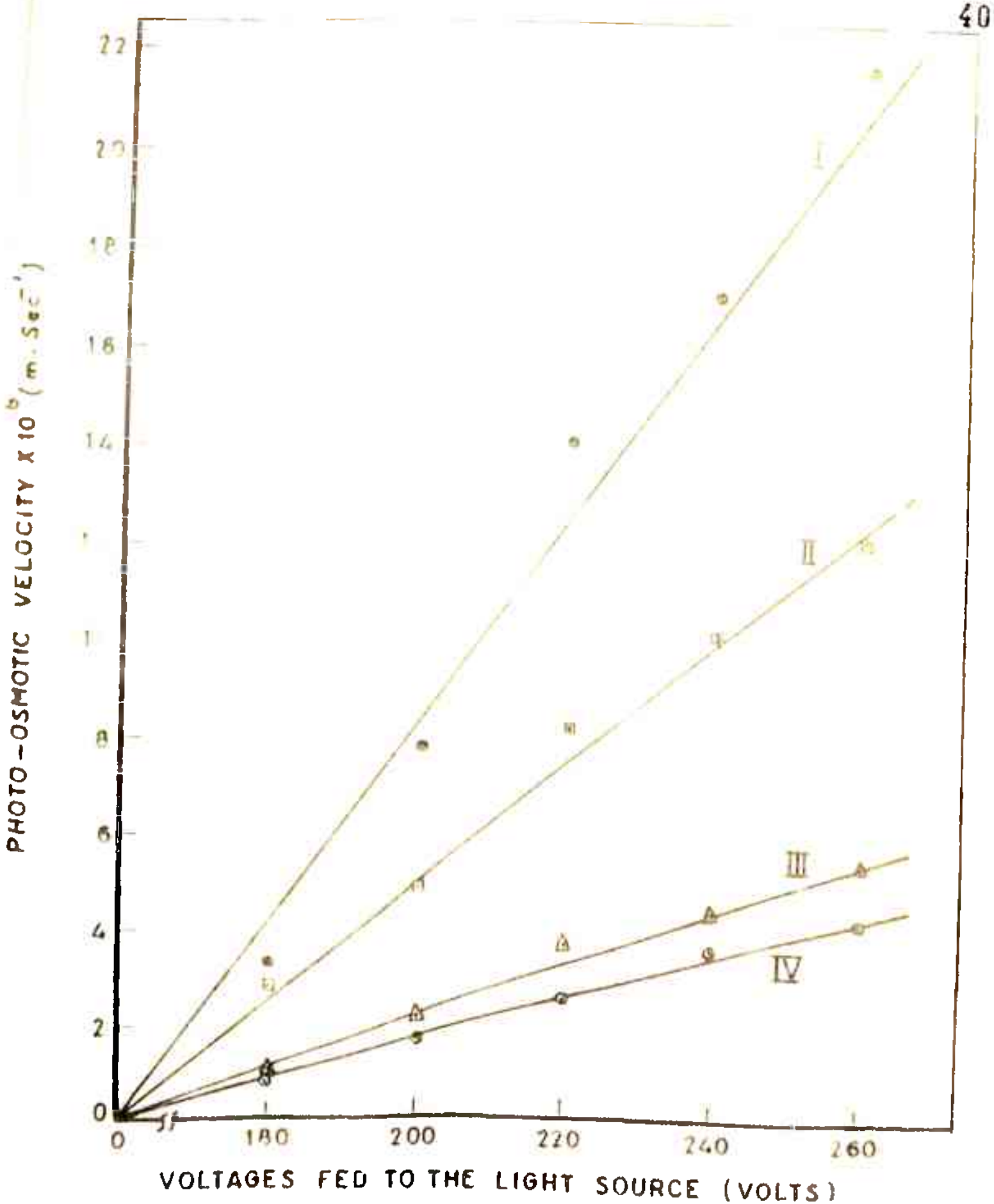

Fig.5. Variation of photo-osmotic velocity with intensity of light. The intensity was varied by feeding different voltages to the light source. Curves I, II, III and IV are for proto-porphyrin, cyanocobalamin, chloroplast extract and haemo-globin respectively.

induced by the light of different wavelengths obtained using different optical filters are recorded in Table VI. The variation of photo-osmotic velocity in case of cyanocobalamin is consistent with its absorption spectrum. The reported[29] absorption maxima for cyanocobalamin are at 278, 361 and 550 nm - the band at 361 nm being more intense than the band at 550 nm. The values of photo-osmotic volume flux at various wavelength ranges show the same gradation (the value of the volume flux at 278 nm could not be obtained due to non-availability of a suitable light source and a suitable filter). In the system containing chloroplast extract, chlorophylls are the main photo-active materials, whose major absorption peaks are at 400 nm and 660 nm[30]. The absorption peak at 400 nm is more intense than the peak at 660 nm. The magnitude of the photo-osmotic velocity at various wavelength ranges (Table VI) shows the same gradation indicating that the photo-osmotic flow is due to the absorption of light by the pigments. Porphyrins are present in both chloroplast extract and haemoglobin. The most intense absorption band for porphyrins is in the region of 400 nm - the soret band[30]. Therefore the observation that in both

Table VI Values of the photo-osmotic velocity at different wavelength ranges.

Wavelength range (nm)	White light	365-445 (Filter No. N-Hg-2)[x]	465-565 (Filter No. B-505)[x]	560-660 (Filter No. B-610)[x]	600-660 (Filter No. N-630)[x]
(a) Chloroplast Extract[†]					
Photo-osmotic velocity Jv x 10^6 (m.sec^{-1})	3.580 ±0.027	2.382 ±0.003	1.315 ±0.001	1.599 ±0.001	2.031 ±0.002
(b) Haemoglobin[†]					
Photo-osmotic velocity Jv x 10^6 (m.sec^{-1})	2.480 ±0.009	1.712 ±0.074	1.383 ±0.027	1.064 ±0.030	1.495 ±0.028
(c) Protoporphyrin[†]					
Photo-osmotic velocity Jv x 10^6 (m.sec^{-1})	14.080 ±0.550	7.400 ±0.290	2.560 ±0.060	3.080 ±0.050	4.580 ±0.190

contd.....

Table VI contd.

(d) Cyanocobalamin†

Wavelength range (nm)	White light	330-430 (Filter No. B-Hg-1)x	465-565 (Filter No. B-505)x	560-660 (Filter No. B-610)x	600-660 (Filter No. N-630)x
Photo-osmotic velocity Jv x 10^6(n.sec^{-1})	8.174 ±0.124	6.732 ±0.086	4.654 ±0.049	4.116 ±0.054	4.515 ±0.045

x Obtained from Photo-volt Corporation, New York.

† The dark compartment contained Fe^{++} ions (1 x 10^{-3}M) and the illuminated compartment contained Fe^{+++} ions (1 x 10^{-3}M).

chloroplast extract and haemoglobin the photo-osmotic velocity is maximum for the wavelength range 365 nm - 445 nm (Table VI) suggests that in these systems absorption of light by porphyrins is responsible for the phenomena of photo-osmosis.

Should it be so protoporphyrin alone should show the phenomena of photo-osmosis. To confirm this suggestion, studies were extended to protoporphyrin. Not only was the phenomenon of photo-osmosis observed, trends in the data were also found to be similar to the trends observed in case of liquid membrane bilayers generated from chloroplast extract. Experiments on protoporphyrin and results obtained therefrom are summarised in the following section.

Experiments on Protoporphyrin:

Protoporphyrin used in these experiments were obtained from Sigma (Cat.No.P5889) and was found to be surface active. The CMC of aqueous protoporphyrin as determined from the variation of surface tension with concentration was found to be 0.7 ppm. Hydraulic permeability data were obtained exactly in the same

manner as described in the section dealing with 'materials and methods'. The hydraulic permeability data showed the same trend as in the case of chloroplast extract confirming the formation of the liquid membrane in series with the supporting membrane. The normalised values of hydraulic conductivity coefficients, L_p/L_p^o, are plotted against concentration in Fig. 3. All other experiments in case of protoporphyrin were also performed in the same way as in the case of chloroplast, haemoglobin. The data obtained therefrom are recorded in Table II to VI and in Fig.5. In the case of protoporphyrin also it was observed that the light induced volume flux was from the illuminated compartment to the dark compartment. It was also observed that (i) as soon as the light was switched on the volume flux was noticed instantaneously, (ii) as soon as the light was switched off the flow stopped completely and (iii) on short circuiting the electrodes E_1 and E_2 (Fig. 2) the light induced volume flux stopped completely. All this leads to the conclusion that in case of protoporphyrin also the observed phenomenon of photoosmosis is due to light induced potential difference

across the membrane. Trends in rest of the data in case of protoporphyrin (Table II to VI and Fig. 5) can be seen to be the same as in the case of chloroplast extract, haemoglobin. The most revealing among these is the trend in the variation of photo-osmotic velocity with wavelength (Table VI). In case of protoporphyrin magnitude of the light induced volume flux, is maximum amongst all the filters used for the one corresponding to the wavelength range 365 - 445 nm (Table VI). This is in conformity with the fact that the reported absorption maxima[30] for protoporphyrin is at 408 nm.

Conclusion:

The present studies indicate that the phenomenon of light induced volume flux which was earlier mimicked on chloroplast - BLMs can also be mimicked on the liquid membrane bilayers generated from chloroplast with trends in the data consistent with those reported on chloroplast - BLMs. This indicates the workability of the liquid membrane bilayers, generated using Kesting's hypothesis, as model systems for biomembranes.

The present studies also indicate that the observed phenomenon of photo-osmosis is due to the absorption of light by porphyrins and porphyrin like structures.

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CHAPTER 3

SURFACE ACTIVITY OF DRUGS

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Formation of cell membranes and location of receptor proteins in the lipid bilayer part of the membranes is all a consequence of surface activity. Hence it is logical to expect that the drugs acting by altering the permeability of cell membranes after interacting with them, should also be surface active in nature. A wide variety of drugs are, infact, known to be surface active in nature[1-7] and as indicated in Chapter 1 there is enough circumstantial evidence to indicate that there might be at least one crucial step common to the mechanism of action of all surface active drugs. Since surface active drugs as per Kesting's hypothesis are capable of generating liquid membranes at the interface, the possibility of the liquid membranes playing a role in the mechanism of action of such drugs cannot be ruled out. Investigations carried out recently on wide variety of drugs[8-12] have strongly indicate in favour of such a possibility. The drugs chosen for these investigations were mostly antagonistic drugs

or those which act by modifying the transport of relevant permeants to the site of action. Nevertheless there is a need to extend the investigations on many more drugs belonging to different pharmacological categories. Studies have therefore been conducted on diazepam, local anaesthetics, propranolol and steroidal drugs. Before an account of these investigations is presented in Chapter 4 a brief survey of relevant literature on surface activity of drugs is in order.

Since excellent reviews[2-4] on the surface activity of drugs are already available, the survey attempted in this Chapter is not intended to be a duplication of earlier efforts. Instead an effort has been made to indicate from the reports on surface activity of drugs, the possibility of surface activity and hence liquid membrane formation by the drugs alone or in association with membrane lipids, contributing to the mechanism of their action.

Tranquillisers:

Phenothiazines are known[13] to reduce membrane permeability at very low concentrations. Ability of

phenothiazinesto reduce water up-take by frog muscle[14,15], inhibition of erythrocyte hemolysis[16-19], inhibition of acetylcholine release[20], inhibition of endogenous amines in various tissues[21-25], inhibition of glycine uptake by brain slices[26] etc. are several examples where the membrane permeability is altered by these drugs.

It is also known[13] that not only phenothiazines but all tranquillisers, irrespective of their chemical nature, lower the surface tension of Ringer solution in close correlation to their clinical potency. It is not surprising therefore that possible adsorption of all of the phenothiazines onto tissue cells may be explained by the physical chemistry involved in air water adsorption[13]. Commenting on the mechanism of alteration of membrane permeability, it is argued[13] that tranquillisers form virtually 'monomolecular films' around cell membrane and reduce transmembrane permeability of solutes.

Interaction of the tranquillisers with insoluble monolayers of lipids, stearic acid etc. constitutes another proof for their surface activity.

Chlorpromazine, chlorpromazine sulfoxide and trifluoperazine have been shown[27,28] to interact with lipid monolayers. Interaction of these drugs with lipids as measured by increase in surface pressure has been shown[27,28] to correlate with their biological activity. Interaction of orphenadrine hydrochloride, chlorpromazine hydrochloride and reserpine with monomolecular films of cholesterol, phosphoglycosides, sphingomyelins, cerebrosides is also documented[29]. Effect of UV radiation on interaction of a series of phenothiazines with dipalmitoyl lecithin films indicated[30,31] that the ability of these drugs to interact with lecithin monolayer may be a measure of their in-vivo membrane penetrating and phototoxic properties.

Structural variation in phenothiazines has been shown[32,33] to alter surface activity, which is evident from the change of critical micelle concentration (CMC) e.g. promazine requires 6 times higher amount of drug to produce the same surface tension as chlorpromazine below CMC and 27 times much higher concentration as compared to triflupromazine[13]. These observations hint at the possibility that

variation in biological activity can be expressed in the form of altered surface activity which indicates that surface activity should have a significant role to play in the mechanism of action of these drugs.

Since phenothiazine-nucleotide interactions are mentioned[34] to be important for its action, the fact[34] that "chlorpromazine can form complexes with adenosine triphosphate and di and mono-phosphate having surface tensions lower than those of the drug alone" appears interesting. It is further indicated[35], that orientation of phenothiazines at the air water interface may reflect qualitative and quantitative differences in their pharmacological actions. In case of phenothiazines, thioxanthene, dibenzocycloheptadiene and dibenzazepines colloidal association and surface activity were found[36] to be dependent on chemical structure of the drug. Direct action of phenothiazine derivative on cat heart causing fall in blood pressure is said[37] to be because of surface activity of the compound.

Haloperidol is known[38] to form a monolayer on water/air or water/lipid interfaces at very low

concentrations. It is further commented[38] that all neuroleptics act like detergents or soaps i.e. they are powerful surface tension lowering agents. A striking correlation between neuroleptic potency and surface tension lowering activity has been indicated. Thus, formation of monolayers on biological structures has been suggested to be a mechanism of neuroleptic action[38].

Tricyclic Antidepressants:

Surface activity of a series of tricyclic antidepressants related to imipramine has been reported[5]. A correlation between surface activity of some of these drugs and their toxicity to chang liver cultures[39,40] and human liver[41] has also been mentioned. Surface activity of a series of drugs which are stimulants or depressants of the nervous system has been reported[42]. It was observed[42] that while the stimulant drug populated in the aqueous bulk phase, the depressant drugs accumulated at the air solution interphase and the depressant effect was found to be correlating with the surface activity of these drugs.

All these evidences support the observations[43] that physico-chemical behaviour of various substituted phenothiazines and other centrally acting drugs[2], especially their surface activity, is important for their action.

Barbiturates:

Butylbarbituric acid[44], pentobarbital, quinalbarbital[45], and mono alkyl, dialkyl barbituric acids[46] have been reported to be surface active. Linear correlation between protein-binding properties and surface activity with apparent partition coefficient has been observed[47]. 'Adsorption free energy' of barbiturates with phospholipid monolayers have been shown[48] to correlate with their nerve-blocking potencies. Changes in ion channels and membrane-bound enzymes as a result of drug-lipid interactions have been indicated[49,50] to be involved in the mechanism of action of barbiturates.

Local Anaesthetics:

A correlation between local anaesthetic activity and surface tension in a series of esters

has been reported[51]. Interaction of local anaesthetic drugs with lipids has been widely investigated[52-55]. For lipids extracted from nervous tissues, penetration of the drugs into the lipid monolayers has been shown to correlate with their nerve blocking potency.

In another study[56], interaction of a series of local anaesthetic drugs with monolayers of dipalmitoyl lecithin indicated that minimum blocking concentration of each of these anaesthetics lowered surface tension of lecthin/water interface by approximately the same amount.

Drugs Related to Acetylcholine:

Choline like compounds have been reported to be surface-active[57-61]. In case of derivatives of papaverine, spasmolytic activity is shown[62] to be proportional to surface activity of these compounds. Curare-like activity of a series of polymethylene-bis-trimethyl ammonium compounds has been shown[63] to be associated with surface activity.

Xanthones and Thioxanthones:

Lucanthone, as anti-schistosomiasis drug, and its derivatives, exhibiting structural similarity to phenothiazines are known[64] to be surface active. A relationship between micellar weight of these compounds and the anti-schistosomiasis activity has been discovered[65].

It is also reported[66] that apparent distribution coefficient of a surface active compound falls steeply above the CMC, as a result of which there is levelling off of the activity above the CMC[2]. In case of some quaternary ammonium salts, micelle formation has been shown to be a limiting factor in their activity[67].

Analgesics:

Surface activity of codeindione, oxycodone[68], antipyrine and its derivatives analgin[69], amidopyrine[70] etc. have been reported. Analgesic action of five narcotic compounds has been observed[71] to correlate with their surface activity at the boundary of air

and benzene solutions of nerve tissue preparations.

β -Blockers:

Surface activity of a series of β -blockers has been reported[72,73]. In another study[74] of β -blockers, properties like effect on myocardial conduction velocity, local anaesthesia have been shown to correlate with surface activity and hydrophobicity.

Antihistamines:

Surface activity of antihistamines[6] and their interaction with dipalmitoyl lecithin monolayers has been reported[75].

Anti-Bacterials:

In case of quaternary ammonium compounds it has been shown[76] that different compounds with equal antimicrobial activity have surface concentrations of the same order of magnitude. This observation is in agreement with another finding[77] that solutions of quaternary ammonium compounds having equal antimicrobial activity have surface tensions of the same order of magnitude.

Tuberculostatics:

Several surface active tuberculostatics based on diaminodiphenyl sulfone have been found[78] to be effective in-vitro in relatively low concentrations. It is suggested[78] that because of their surface activity, these molecules are adsorbed at the bacterial surface with the sulphone portion of the molecule embedded in the cell and polyoxyethylene chains oriented out wards. In another report[79], antitubercular activity of the drugs is attempted to be related to their configuration at air/water interface.

The fact that such a wide variety of drugs are surface active and a correlation between surface activity and biological activity is indicated, hints at the possibility that a common mode of action may be operative in the mechanism of action of these drugs. For surface active substances, reduction of surface tension is accompanied by formation of a surface layer which is complete at or above CMC. Hence the surface active drugs are expected to form a "liquid membrane" interposed between the biological membrane and its relevant permeants. Several factors e.g. orientation of the surface active drug with respect

to biological membrane, active interaction of the drug with biomembrane, nature of interaction within the surface active drugs, presence of double layer around the drug liquid membrane may influence its permeability characteristics. However one possibility appears quite obvious i.e. access of the permeant to the 'receptors' located on the biomembrane will certainly be altered by presence of a liquid membrane, generated by the drug.

It has been indicated[80] that "Aqueous films formed from solutionsof surface-active drugs might be sensitive tools with which to study the interactions of the drugs with ions, especially those of biological importance and with which to obtain a more quantitative insight into the behaviour of the drug molecules at surfaces".

It has also been commented[2] that "Surface tension reduction is only a symptom of many physicochemical attributes and much work still remains to be done before surface activity per se can be a reliable guide to biological activity in a homologus series". The liquid membrane hypothesis for

drug action appears to be a positive step in this direction.

The liquid membranes generated by the drugs would alter transport of various other permeants also in addition to the permeants relevant for the specific biological effect of the drug. This in turn indicates existence of multiplicity of biological effects of surface active drugs. Multiple effects have infact been observed in the case of several surface active drugs[81,82].

Fig. 1 reproduced from Florence's excellent review[2] article clearly depicts interrelation between several categories of surface active drugs.

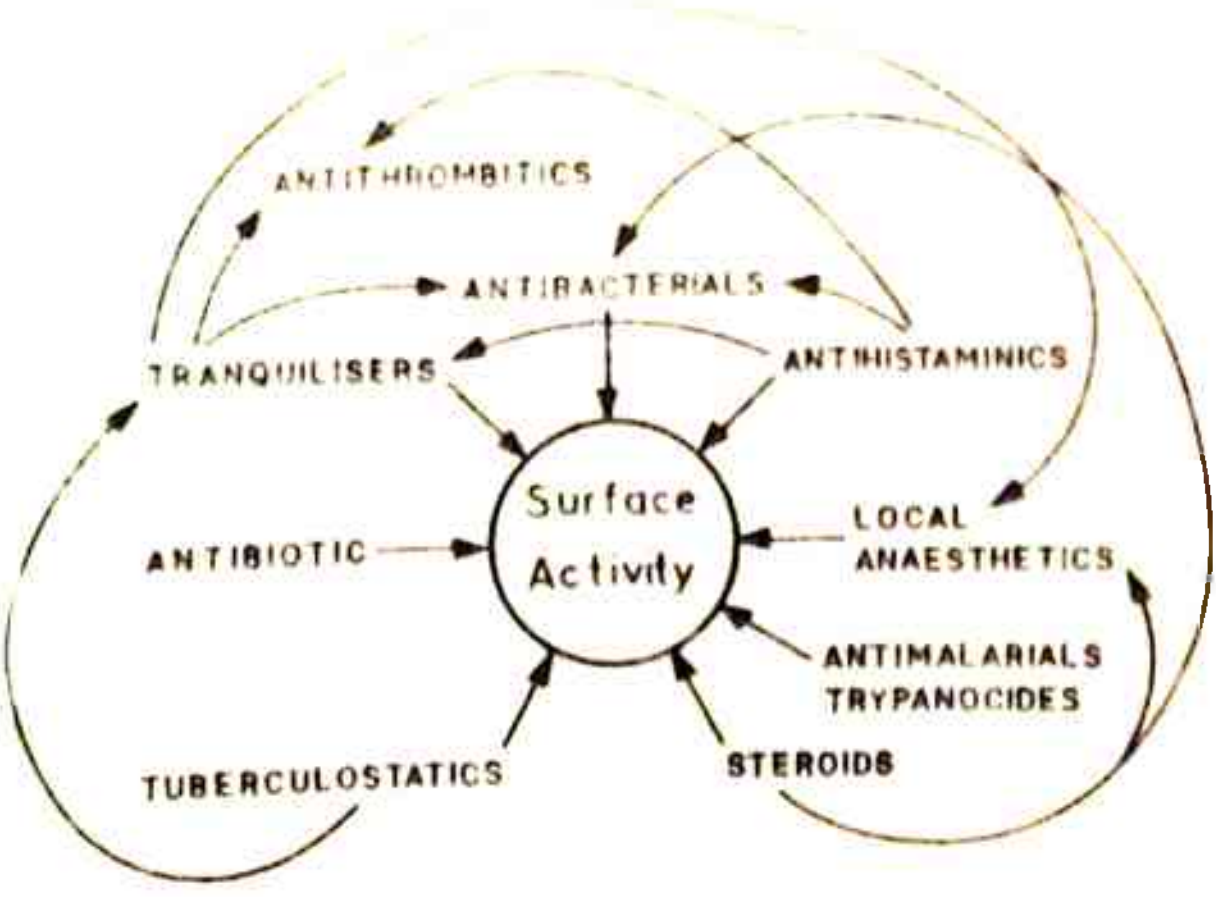

Fig. 1. Diagram showing inter-relationship between drugs showing surface activity. Arrows joining to centre indicate that that class of drug has exhibited activity. Arrows linking different pharmacological types show secondary activity of some of the members of the class, e.g., some steroids have local anaesthetic activity; some tuberculostatics have shown tranquillising activity. It has been suggested that some diuretics are membrane-active but there appears to be no evidence of their surface activity.

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CHAPTER 4

LIQUID MEMBRANE PHENOMENON IN DRUG ACTION

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In this Chapter are reported studies on four different categories of drugs with a view to exploring the role of liquid membranes generated by them in the mechanism of their action. These are

(a) Local anaesthetics:

Procaine, Dibucaine, Lidocaine and Tetracaine (all hydrochloride salts).

(b) Tranquillisers:

Diazepam.

(c) Steroidal drugs:

Testosterone propionate, Ethinylestradiol and Hydrocortisone acetate.

(d) β-Blockers:

Propranolol.

Two kinds of data were obtained to investigate the role of liquid membranes generated by the drugs in the mechanism of their action. The data on hydraulic

permeability in presence of the drugs were utilised to demonstrate the formation of a liquid membrane in series with a supporting membrane and the data on the solute permeability of relevant permeants in the presence of the liquid membranes thus generated were utilised to gain information on the role of liquid membranes, in the mechanism of their action. In all these experiments a cellulosic membrane/aqueous interface has been chosen as site for the formation of liquid membranes. This has been purposely done so that the active interaction of the drugs with biomembranes as a cause for the modification in the permeabilities is totally ruled out and the contribution of passive transport through the liquid membranes is high lighted.

The studies conducted on the various drugs belonging to different categories listed above are described in the following sub-sections.

STUDIES ON LOCAL ANAESTHETICS

Most of the useful local anaesthetics contain both a hydrophilic and a hydrophobic part in their

structure[1] and hence are surface active in nature. They act by modifying the permeabilities of nerve cell membranes to sodium and potassium ions. For the present study, four local anaesthetic drugs namely procaine, lidocaine, tetracaine and dibucaine have been chosen. Existence of a liquid membrane generated by each of these drugs at the interface has been demonstrated. Data on transport of sodium and potassium ions through the liquid membranes generated by these drugs in series with supporting membrane has been obtained to gain information on the contribution of the liquid membrane in the mechanism of action of the drugs. Since local anaesthetics are known to interact with membrane lipids[2] the studies have been extended to the liquid membranes generated by lecithin-cholesterol-local anaesthetic drugs mixtures. In these experiments a cellulose nitrate microfiltration membrane/aqueous solution interface was chosen as site for the formation of liquid membranes.

Materials and Methods:

Procaine hydrochloride (Farbwerke Hoechst A.G. Germany), Lidocaine hydrochloride (John Baker Inc. Colorado, USA), Tetracaine hydrochloride (John Baker Inc. Colorado, USA), Dibucaine hydrochloride (John Baker Inc. Colorado, USA), Lecithin (Patel Chest Institute, Delhi), Cholesterol (Centron Research Laboratories, Bombay), Chlorides of sodium and potassium (both BDH Analytical reagents) and distilled water, distilled once over potassium permanganate in all pyrex glass still were used in present experiments.

The critical micelle concentration (CMC) of aqueous solutions of procaine hydrochloride, lidocaine hydrochloride, tetracaine hydrochloride and dibucaine hydrochloride were determined from the variation of surface tensions with concentrations. These are recorded in Table I. The surface tensions were measured using a surface tensiomat (Fisher Tensiomat Model 21).

Table I Critical micelle concentration (CMC) and Minimum blocking concentration (MBC) of local anaesthetics.

	Tetracaine hydrochloride	Dibucaine hydrochloride	Lidocaine hydrochloride	Procaine hydrochloride
CMC	12.212 μM	5.640 μM	1.160 mM	5.000 mM
MBC	10.000 μM[a]	5.000 μM[a]	1.170 mM[b]	4.460 mM[a]

a ref. 15.

b calculated on the basis of anaesthetic potency ref. 2.

The all glass cell used for transport studies is described in Fig. 1 which has been well labelled to make it self explanatory. A Sartorious cellulose nitrate microfiltration membrane (Cat. No. 11307, bubble point 3.7 bar and average pore size 0.2 μm) of thickness 1×10^{-4} m and area $5.373 \times 10^{-5} m^2$, which acted as a support for the liquid membrane, separated the transport cell into two compartments C and D.

For measurements of hydraulic permeability in presence of local anaesthetic drugs, aqueous solutions of the drugs of varying concentrations were filled in the compartment C of the transport cell and the compartment D was filled with distilled water. Known pressures were applied on the compartment C by adjusting the pressure head and the consequent volume flow was noted by noting the advancement of the liquid meniscus in the capillary L_1 L_2 using a cathetometer reading upto 0.001 cm and a stop watch reading upto 0.1 sec. During the volume flux measurements the solution in the compartment C was kept well stirred and the

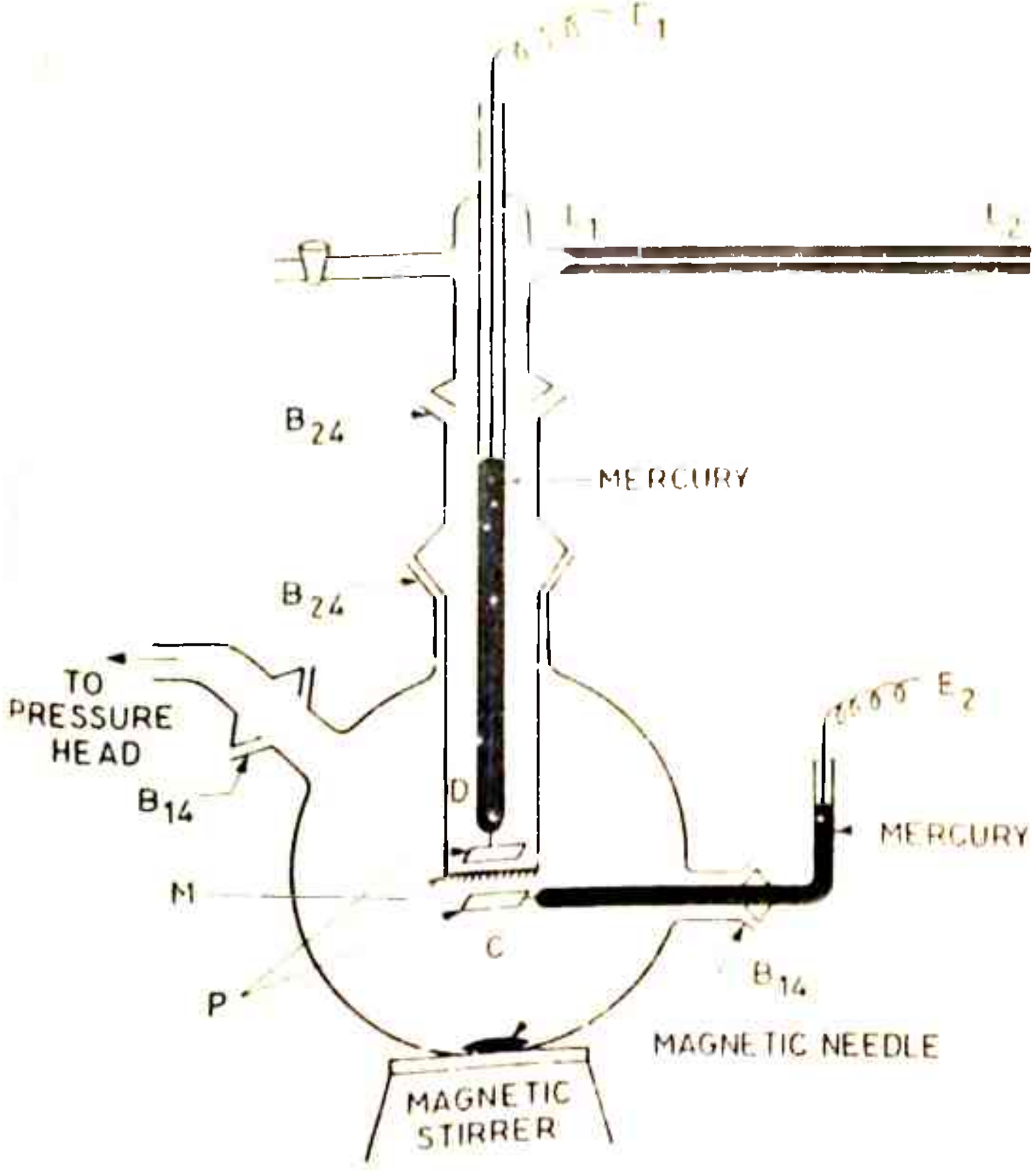

Fig. 1. The all glass transport cell. M - supporting membrane (cellulose acetate microfiltration membrane cat.no.11307 of thickness 1×10^{-4}m and area 5.373×10^{-5}m^2), P -bright platinum electrodes, E_1,E_2 electrode terminals, L_1 L_2 - capillary of length 17 cm and diameter 1.18×10^{-1}cm.

electrodes A_1 and A_2 were short circuited so that the electro-osmotic back flow due to the streaming potential which might develop across the membrane does not become a serious disturbing factor. The concentration ranges of the local anaesthetic drugs chosen were such that one obtains data on both, lower and higher sides of CMC of the drugs. Hydraulic permeability measurements were also carried out at various concentrations of local anaesthetic drugs in presence of lecithin-cholesterol mixture of fixed composition. For this solutions of various concentrations of local anaesthetic drugs prepared in an aqueous solution of lecithin-cholesterol mixture which was 15.542 ppm with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol were filled in the compartment C of the transport cell (Fig. 1) and the compartment D was filled with distilled water. This particular composition of lecithin-cholesterol mixture was chosen because it has been shown[3] that at this composition the liquid membrane generated by lecithin is saturated with cholesterol and completely covers the supporting membrane.

For the measurement of solute permeability (ω) of sodium and potassium ions, two sets of experiments were performed. In the first set of experiments, the compartment C of the transport cell (Fig. 1) was filled with a solution of the electrolyte (sodium chloride or potassium chloride) of known concentration prepared in the aqueous solution of known concentration of the local anaesthetics and compartment D was filled with distilled water. In the second set of experiments aqueous solution of known concentrationof the permeants (sodium chloride or potassium chloride) was filled in the compartment C and aqueous solution of known concentrations of the drug was filled in the compartment D of the transport cell (Fig. 1). In control experiments, however no drug was used. Concentration of the drugs in these experiments was always higher than their CMC to ensure complete coverage of the supporting membrane with the liquid membrane generated by the drugs. Because of surface active nature, the hydrophobic portion of the drug molecules will be preferentially oriented towards the hydrophobic supporting membrane and the hydrophilic parts of the drug molecules will be drawn outwards away from it. Thus, in the first

set of experiments where both, the permeants and the drug are present in the same compartment, the permeants would face the hydrophilic surface of the liquid membrane generated by the drug while in the second set of experiments the permeants would face the hydrophobic surface of the drug liquid membrane. Initial concentrations of sodium and potassium ions in the solute permeability experiments were chosen so as to correspond to the concentrations of the ions in the neighbourhood of nerve cells. The condition of no net volume flux ($J_v = 0$) was imposed on the system by adjusting the pressure head attached to the compartment C of the transport cell (Fig. 1) so that the liquid meniscus in the capillary L_1 L_2 remained stationary. After a known period of time the concentration of the permeant in the other compartment was measured. The amount of the permeant transported divided by the time and the area of the membrane gave the value of the solute flux (J_s). The value of the solute permeability (ω) was estimated using the definition[4,5]

$$\left(\frac{J_s}{\Delta \pi}\right)_{J_v = 0} = \omega \qquad (1)$$

where $\Delta\pi$ is the osmotic pressure difference. The value of $\Delta\pi$ used in the calculation of ω was the average of the $\Delta\pi$ values at the beginning ($t = 0$) and at the end of the experiment.

Values of (ω) for sodium and potassium ions were also estimated in presence of lecithin-cholesterol-local anaesthetic drug mixtures. Since the transport of cations was observed to be impeded more when the permeants face the hydrophobic surface of the liquid membrane, the values of (ω) in presence of lecithin-cholesterol-drug mixtures were measured in the second set of experiments only. For this the solution of local anaesthetic drugs prepared in the aqueous solution of lecithin-cholesterol mixtures of composition 15.542 ppm with respect lecithin and 1.175×10^{-6}M with respect to cholesterol was filled in the compartment D of the transport cell (Fig. 1) and aqueous solution of the permeants (sodium or potassium ions) were filled in the compartment C. The concentrations of the local anaesthetic drugs in these experiments were those at which the liquid membrane generated by lecithin-cholesterol mixture

gets saturated by the drugs. These concentrations were derived from the hydraulic permeability data in presence of lecithin-cholesterol-drug mixtures.

The amount of sodium and potassium ions transported in a given time, to the compartment D were estimated using a flame photometer (CSIO AIMIL-PRI).

All measurements were made at constant temperature using a thermostat set at 40 ± 0.1°C.

Results and Discussion:

The hydraulic permeability data at various concentrations of the drugs, in the case of all the four local anaesthetic drugs, were found to obey the linear relationship,

$$Jv = L_p \Delta P \qquad (2)$$

where L_p is the hydraulic conductivity coefficient and ΔP is the applied pressure difference. The values of L_p calculated from the slopes of the Jv vs. ΔP plots show a progressive decrease as concentration of the drug is increased. This trend

in the value of Lp continues upto the CMC beyond which the decrease in the values of Lp is only marginal. The normalised values of the hydraulic conductivity coefficients i.e. the values of L_p/L_p^o, where L_p^o is the value when no drug was used, are plotted against concentration of the drug in the Fig. 2. The observed gradation in the values of L_p (Fig. 2) is in keeping with Kesting's hypothesis[6-8], according to which as concentration of the surfactant is increased the supporting membrane gets progressively covered with the surfactant layer liquid membrane and at the CMC it is completely covered. The marginal decrease beyond the CMC is most probably due to an increase in density of the liquid membrane[8].

Analysis of the flow data in the light of mosaic membrane model[9,10] furnishes further evidence in favour of the existence of liquid membrane in series with the supporting membrane. Following the arguments and the procedure given in Chapter 2 (pages 24-26) it is possible to compute the values of Lp at various concentrations of the surfactant below its CMC from the experimentally determined

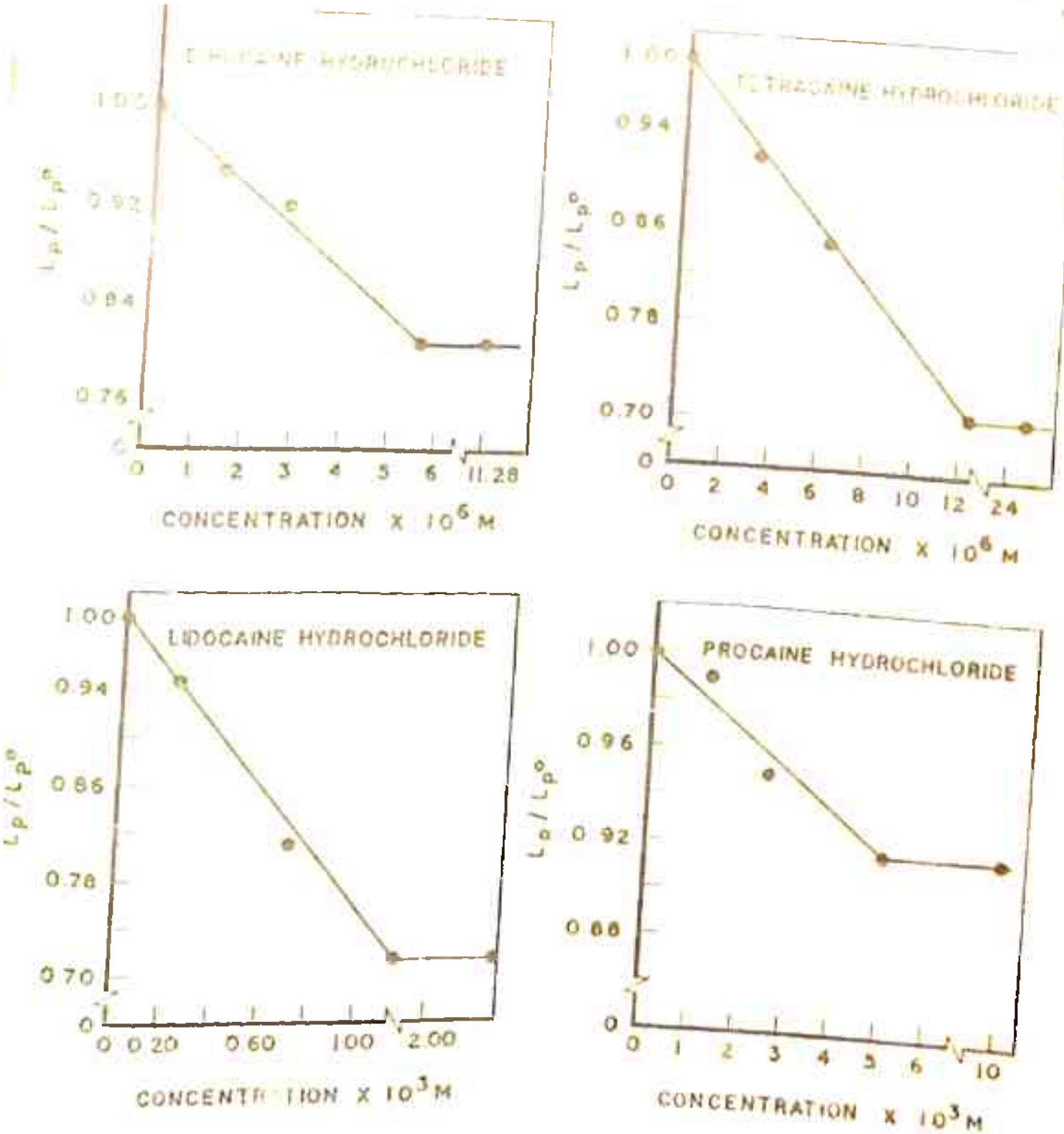

Fig. 2. Values of L_p/L_p^o at various concentrations of Local Anaesthetic drugs.

values of L_p at 0 and the CMC of the surfactant. The values L_p thus computed at various concentrations of tetracaine hydrochloride below its CMC are in good agreement with the experimentally determined values (Table II) confirming the formation of a liquid membrane at the interface. Analysis of the hydraulic permeability data in case of other local anaesthetic drugs namely, procaine, dibucaine, and lidocaine also gave similar results.

The hydraulic permeability data at various concentrations of local anaesthetic drugs in presence of lecithin-cholesterol mixtures was also found to be in accordance with equation (2). The normalised values of hydraulic conductivity coefficient i.e. the values of (L_p/L_p^o), where L_p^o is the value of L_p in presence of the lecithin-cholesterol mixture only are plotted against the concentrations of the drugs in Fig. 3. The decrease in the value of (L_p/L_p^o) is indicative of incorporation of the drugs in the liquid membrane generated by the lecithin-cholesterol mixture. The concentration of the drug beyond which the values of (L_p/L_p^o) become more or less constant

Table II Values of L_p at various concentrations of tetracaine hydrochloride.

Concentration x 10^6M	0.0	3.053 (0.25CMC)	6.106 (0.5CMC)	12.212 (CMC)	24.424
L_p^* x 10^8($m^3s^{-1}N^{-1}$)	5.021 ±0.077	4.615 ±0.116	4.284 ±0.144	3.560 ±0.230	3.550 ±0.290
L_p^{**} x 10^8($m^3s^{-1}N^{-1}$)	-	4.655 ±0.114	4.291 ±0.154	-	-

* Experimental values.

** Computed values using mosaic model.

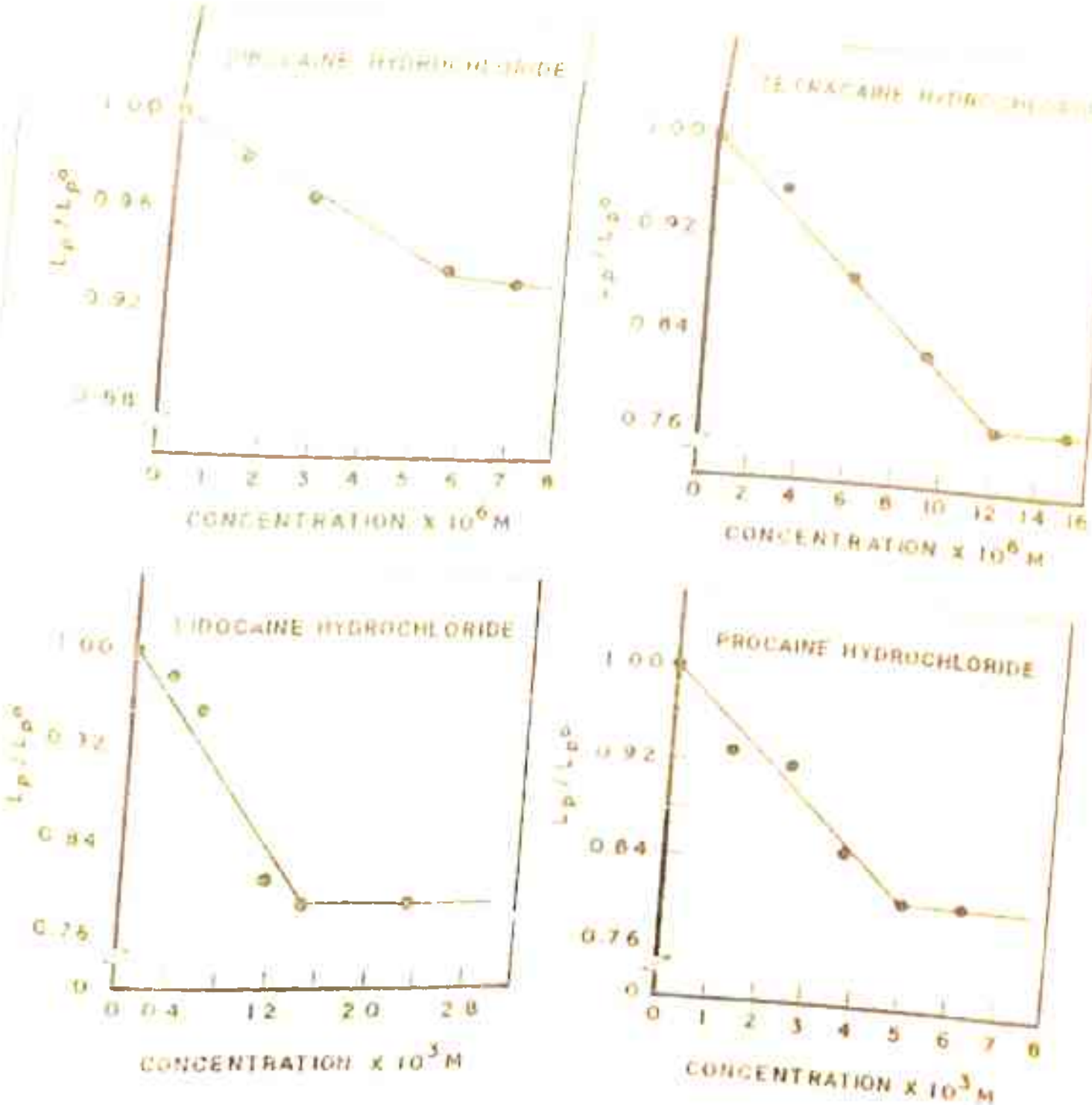

Fig. 3. Values of L_p/L_p^o at various concentrations of Local Anaesthetic drugs, in presence of Lecithin-cholesterol mixture, ~~solution~~ of fixed composition - 15.542 ppm with respect to Lecithin and 1.175×10^{-6} M with respect to Cholesterol.

is the concentration at which the liquid membrane generated by the lecithin-cholesterol mixture is saturated with the drug. The concentrations at which tetracaine, lidocaine, dibucaine and procaine saturated the liquid membrane generated by the lecithin-cholesterol mixtures were found to be 12.21×10^{-6}M, 1.16×10^{-3}M, 5.64×10^{-6}M and 5.0×10^{-3}M respectively.

In order to ascertain the location of the drugs in the lecithin-cholesterol liquid membrane surface tensions of the solutions of various concentrations of drugs prepared in the aqueous solution of lecithin-cholesterol mixture of fixed composition of 15.542 ppm with respect to lecithin and 1.175×10^{-6}M with respect to cholesterol, were measured. The surface tension of the aqueous solution of lecithin-cholesterol mixture showed further decrease with increase in concentration of the local anaesthetics. This indicates that the drugs penetrate the liquid membrane generated by the lecithin-cholesterol mixture and reach the interface.

Role of Liquid Membrane in Local Anaesthesia:

The critical micelle concentrations of the local anaesthetic drugs as determined in the present experiments are more or less equal to their minimum blocking concentrations (MBC) (Table I). Since according to Kesting's hypothesis[6-8] CMC is the concentration at which the interface gets completely covered with the liquid membrane, it appears prima facie that the liquid membranes generated by the local anaesthetic drugs at the site of their action may have a role to play in the local anaesthetic action. This is further supported by the gradation in the binding of local anaesthetics to nervous and other tissues[11]. The binding affinities of these drugs increase in the following order

procaine < lidocaine < tetracaine < dibucaine.

The fact that the CMC values of these drugs are in the reverse order i.e. dibucaine < tetracaine < lidocaine < procaine (Table I) is also indicative of the role of liquid membrane phenomena in their action. Higher is the value of CMC, higher is the

concentration required to generate a complete liquid membrane at the site of action. As reported by Skou[2] relative anaesthetic potencies of procaine, lidocaine and tetracaine are in the following order

tetracaine > lidocaine > procaine.

The above gradation is in agreement with the descending order of the CMC value of these drugs (Table I) - the lower is the CMC value more potent is the drug. This again indicates the role of liquid membrane phenomena in local anaesthesia.

The local anaesthetic action is linked with the inhibition of the transport of the sodium and potassium ions. The normalized values of the solute permeabilities i.e. the value of (ω/ω°), ω° being the value for the control experiment where no drug was used, for sodium and potassium ions in presence of the local anaesthetic drugs alone and also in presence of lecithin-cholesterol-local anaesthetic drug mixture, are recorded in Table III. A perusal of Table III reveals that the permeabilities of sodium and potassium ions are inhibited

Table III Values of normalised permeabilites $r = (\omega/\omega^*)$ for sodium and potassium ions in presence of local anaesthetic drugs and lecithin-cholesterol-local anaesthetic mixtures.

	Sodium			Potassium		
	r^x	r^{xx}	r^{xxx}	r^x	r^{xx}	r^{xxx}
Dibucaine Hydrochloride	0.850	0.783	0.846	0.648	0.504	0.798
Tetracaine Hydrochloride	0.788	0.605	0.919	0.680	0.633	0.765
Lidocaine Hydrochloride	0.960	0.938	0.992	0.700	0.447	0.850
Procaine Hydrochloride	0.958	0.935	0.952	0.897	0.684	0.950

x Both permeants and the drugs in the compartment C and water in the compartment D.

xx Aqueous solution of the drugs in the compartment D and aqueous solution of permeants in the compartment C.

xxx Lecithin-cholesterol-drug mixture in the compartment D and permeants in the compartment C.

in both the sets of experiments. However the inhibition is maximum in the second set of experiments where the permeating cations face the hydrophobic surface of the liquid membrane generated by the local anaesthetic drugs alone.

Local anaesthetic drugs are known[12] to reduce permeability of resting nerve to potassium as well as to sodium ions. They also reduce permeability to sodium and potassium ions in the membranes of muscle both in the resting state and during the generation of an action potential[13]. The net effect depends however on the extent to which cationic and non-ionic forms of the local anaesthetics are available. In case of procaine, it is shown[14] that while uncharged form causes decrease in resting sodium conductance, the charged form decreases the resting potassium conductance. Thus the reduced permeability of both sodium and potassium ions as observed in the present experiments (Table III) is in conformity with the literature reports related to biological cells.

Ability of local anaesthetics to block nerve impulse conduction has been shown to correlate with

their interaction with the lipid monolayers[15]. Both, the ability to interact with the lipid films and the ability to block nerve conduction are reported to be pH dependent[12]. It has also been observed particularly in the case of procaine, that the drug penetrates only into the ionic region of the lipid monolayers[16]. The action of local anaesthetics is known to be short lived and reversible in nature. This appears to be on account of the fact that interaction of local anaesthetics with cell membranes is limited only to the ionic surface of the membranes[16,17].

In the model proposed by Lee[18] for action of local anaesthetics sodium channels are postulated to be surrounded by an annulus of lipid which is in the crystalline or gel state. It is the rigidity of the surrounding lipid micro-environment that keeps the sodium channel open. Addition of local anaesthetics triggers a change in the surrounding lipids to the fluid liquid-crystalline phase allowing the sodium channel to close with resulting local anaesthesia. Drop in phase transition temperature due to the addition of local anaesthetics is cited[19] as an

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evidence for fluidisation. Since in the lowering of phase transition temperatures head group interactions are known[20] to play an important role it appears that polar head of local anaesthetic molecules interact, from within the channel, with polar heads of lipids surrounding the channel and fluidise them. As a consequence of loosening of lipid micro-environment the channel is filled up with a hydrophobic core consisting mainly of hydrophobic moities of local anaesthetic molecules impeding the transport of sodium ions.

In the present experiments however resistance offered to the transport of cations in presence of lecithin-cholesterol-local anaesthetic drug mixture is less than that in presence of the local anaesthetic drugs alone (Table III) in the second set of experiments. This is because the local anaesthetic drug molecules are incorporated within the liquid membrane generated by lecithin-cholesterol mixtures and their hydrophobic moities are less available to approaching cations to impede their transport. Since planar configuration of lipid films is known to be more stable than circular configuration[21] the expected fluidisation leading to

of several drugs with phospholipid monolayers are available in literature[23]. The studies have therefore been extended to the liquid membranes generated by lecithin-cholesterol-diazepam mixture. The transport data indicate that the liquid membrane phenomena may make a notable contribution to the actions of diazepam. In these experiments also, a cellulose nitrate microfiltration membrane/aqueous interface has been deliberately chosen as site for the formation of liquid membranes so that active interaction of the drug with components of biomembranes is totally ruled out and the contribution of the passive transport through the liquid membrane is highlighted.

Materials and Methods:

Diazepam (Ranbaxy Laboratories, Delhi), glycine (BDH), GABA (gamma amino butyric acid, BDH), methyl cellosolve (Riedel-De Haenag Seelze-Hannover, Germany), ninhydrin (Fisons Philadelphia, USA), hydrindantin (Koch-Light Laboratories Ltd.), lecithin (Patel Chest Institute, Delhi), cholesterol (Centron Research Laboratories, Bombay), and distilled water, distilled once over potassium permanganate in an all-pyrex glass still were used in the present experiments.

The critical micelle concentration (CMC) of aqueous diazepam was determined from the variation of surface tension with concentration. The surface tensions were measured using a Fisher Tensiomat model 21. To prepare aqueous solutions of diazepam necessary volume of ethanolic solutions of known concentration of the drug was added with constant stirring to the aqueous-phase. In the aqueous solutions of diazepam thus prepared, the final concentration of ethanol was never allowed to exceed 0.1% (by volume) because it has been shown by a control experiment that a 0.1% solution of ethanol in water did not lower the surface tension of water to any measurable extent. The CMC value of aqueous diazepam was found to be 1×10^{-4}M.

For hydraulic permeability measurements two sets of experiments were performed. In one set of experiments aqueous solutions of diazepam of various concentrations ranging from 0 to 2×10^{-4}M were filled in the compartment C of the transport cell (Fig. 1) and the compartment D was filled with water. The concentration range from 0 to 2×10^{-4}M was chosen to get data on both the lower and the

higher side of the CMC of the diazepam. In another set of experiments solutions of various concentrations of diazepam prepared in an aqueous solution of lecithin-cholesterol mixture which was 15.542 ppm with respect to lecithin and 1.175×10^{-6}M with respect to cholesterol, were filled in the compartment C and the compartment D of the transport cell was filled with water. As already mentioned in the previous section this particular composition of lecithin-cholesterol mixture was chosen because it has been shown in an earlier study[3] that at this composition the liquid membrane generated by lecithin is saturated with cholesterol and completely covers the supporting membrane. The procedure for hydraulic permeability measurements was the same as described in the previous section (pages 80-82).

Solute permeabilities (ω) for glycine and GABA were measured in presence of both diazepam and the lecithin-cholesterol-diazepam mixture. For measurements of ω in presence of diazepam two sets of experiments were performed. In the first

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Solute permeabilities (ω) for glycine and GABA were measured in presence of both diazepam and the lecithin-cholesterol-diazepam mixture. For measurements of ω in presence of diazepam two sets of experiments were performed. In the first

set of experiment solution of the permeants - glycine and GABA, prepared in aqueous solution of known concentration of diazepam was filled in the compartment C and the compartment D was filled with water. In the second set of experiments the aqueous solution of diazepam was filled in the compartment D and the aqueous solution of the permeant was filled in the compartment C. In control experiments, however, no diazepam was used. The concentration of diazepam used in these experiments was 1.6×10^{-4}M which is well above its CMC. Similar sets of experiments were carried out for ω measurements in presence of lecithin-cholesterol-diazepam mixtures. The diazepam concentration in these experiments was 0.75×10^{-4}M, the concentration at which the liquid membrane generated by lecithin-cholesterol mixture gets saturated with diazepam. The procedure adopted for ω measurements was similar to the one described in the previous section on local anaesthetics (pages 83-86).

The amount of glycine and GABA transported to the compartment D were estimated from the amount of their reaction products with ninhydrin measured

Spectrophotometrically at 570 nm[24] using a Bausch and Lomb Spectronic 20 spectrophotometer.

All measurements were carried out at constant temperature using a thermostat at 40 ± 0.1°C.

Results and Discussion:

The hydraulic permeability data at various concentrations of diazepam were found to be in accordance with equation (2). The normalised values of the hydraulic conductivity coefficient, (L_p/L_p^o), are plotted against the concentrations of diazepam in Fig. 4. The trend in Fig. 4 is in accordance with Kesting's hypothesis[6-8] and is indicative of the progressive coverage of the supporting membrane by the liquid membrane generated by diazepam - at the CMC, the supporting membrane is completely covered by the drug liquid membrane. Analysis of the flow data in the light of the mosaic membrane model[9,10] further confirms the formation of diazepam liquid membrane in series with the supporting membrane. The values of L_p computed using mosaic model at various concentrations

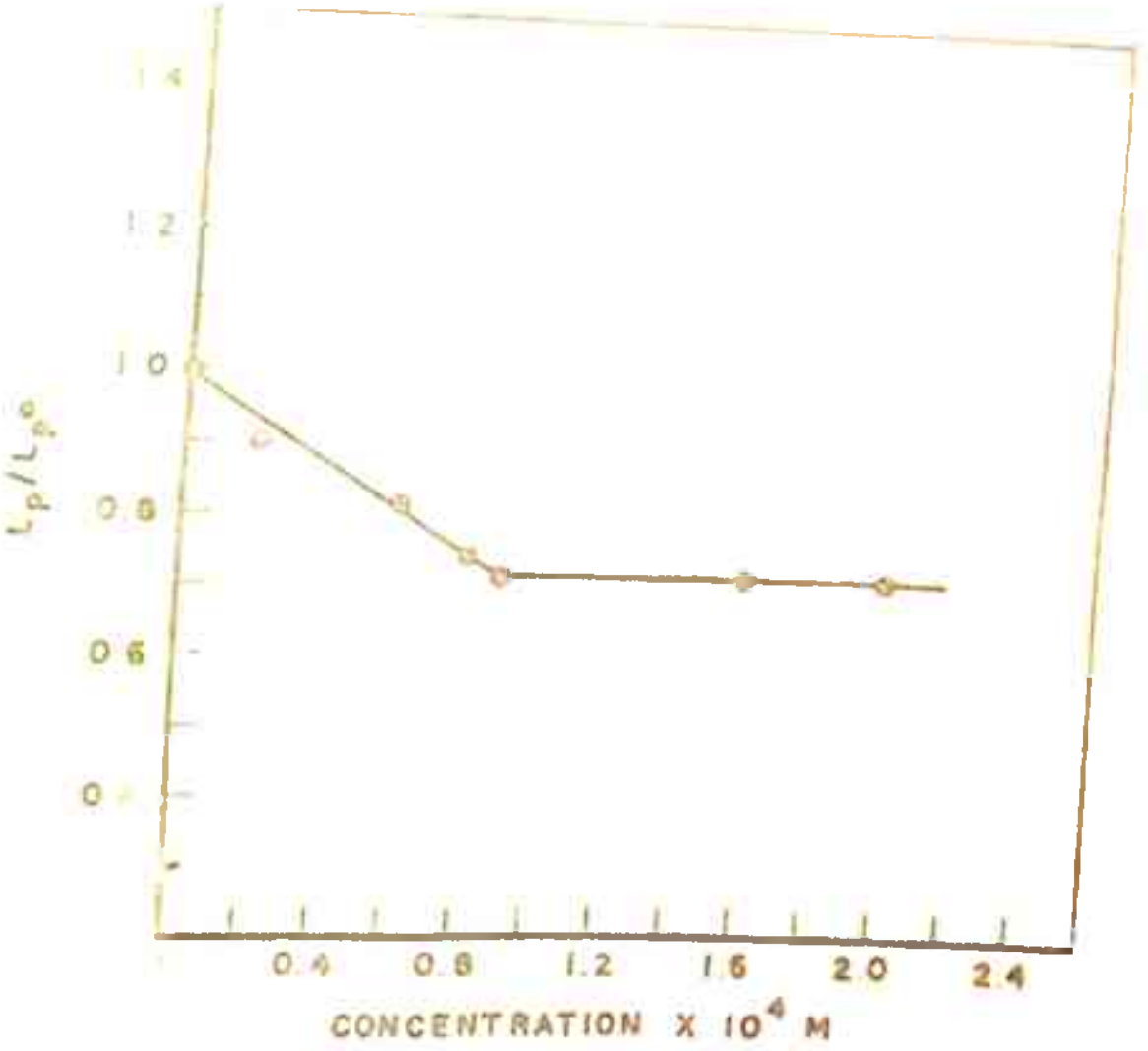

Fig. 4. Variation of L_p/L_p^o with concentration of diazepam.

below the CMC of diazepam match favourably with the experimentally determined values (Table IV).

The permeability data for the other set of experiments where solutions of various concentrations of diazepam prepared in the aqueous solution of the lecithin-cholesterol mixtures were filled in the compartment C of the transport cell and distilled water was filled in the compartment D were also found to be in accordance with the linear equation (2). The normalised values of the hydraulic conductivity coefficients are plotted in Fig. 5. The values of (L_p/L_p°) show a decrease with increasing concentration of diazepam upto 0.75×10^{-4}M beyond which they become more or less constant. This indicates that diazepam gets incorporated within the liquid membrane generated by the lecithin-cholesterol mixture and when its concentration equals 0.75×10^{-4}M the lecithin-cholesterol liquid membrane is saturated with diazepam. In order to ascertain whether diazepam reaches straight upto the interface or not surface tensions of the solutions of various concentrations

Table IV Values of L_p at various concentration of diazepam.

Concentration x 10^4M	0.0	0.20 (0.20MC)	0.60 (0.60MC)	0.80 (0.80MC)	1.00 (1CMC)	1.60	2.00
L_p^* x 10^8($m^3 s^{-1} M^{-1}$)	2.234 ±0.139	2.014 ±0.027	1.830 ±0.052	1.660 ±0.042	1.590 ±0.020	1.590 ±0.015	1.590 ±0.020
L_p^{**} x 10^8($m^3 s^{-1} M^{-1}$)	-	2.103 ±0.113	1.484 ±0.068	1.719 ±0.044	-	-	-

* Experimental value.

** Computed values using mosaic model.

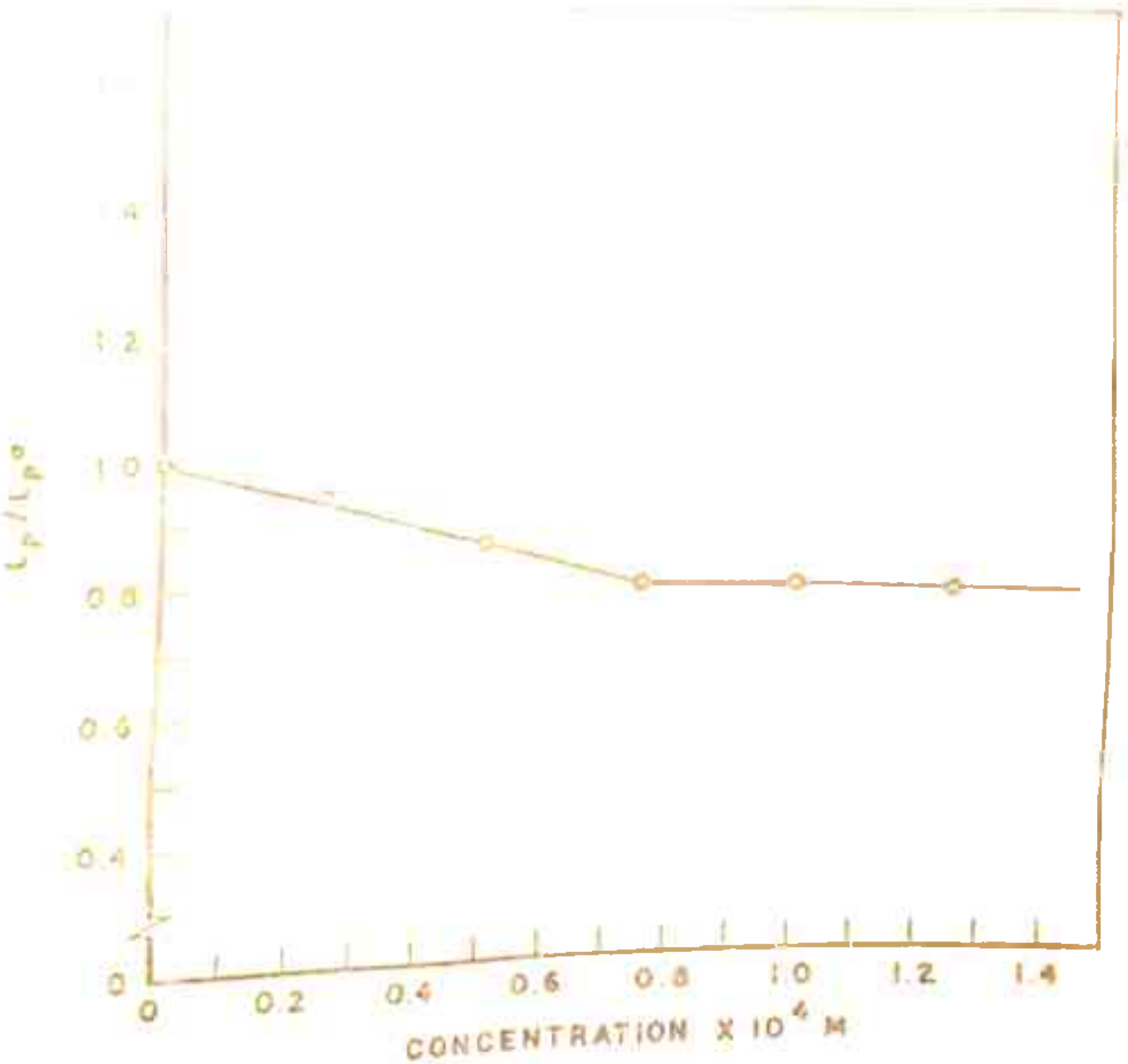

Fig. 5. Variation of L_p/L_p^o with concentration of Diazepam, in presence of Lecithin-cholesterol mixture of fixed composition - 15.542 ppm with respect to Lecithin and 1.175×10^{-6}M with respect to Cholesterol.

of diazepam prepared in the aqueous solutions of lecithin-cholesterol mixtures of fixed composition - 15.542 ppm with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol, were measured. The surface tension of aqueous solutions of the lecithin-cholesterol mixture showed a further decrease with the increase in concentration of diazepam upto 0.75×10^{-4}M. This indicates that diazepam penetrates the liquid membrane generated by the lecithin-cholesterol mixture and reaches upto the interface.

The normalised values of solute permeabilities (ω/ω°) in presence of diazepam (Table V) indicate that the diazepam liquid membrane in both the sets of experiments offers resistance to the transport of the amino acids. Because the hydrophobic ends of diazepam will be preferentially oriented towards the hydrophobic supporting membrane, in the first set of experiments the permeants will face the hydrophilic surface of the liquid membrane generated by diazepam. In the second set of experiments, however, where diazepam is present in the compartment D and the permeants in the compartment C (Fig. 1) the liquid membrane

Table V Values of normalised permeabilities $r = (\omega/\omega^{\circ})$ of amino acids in presence of diazepam[a] and lecithin-cholesterol-diazepam mixtures[b].

	r^{x}	r^{xx}	r^{xxx}	r^{xxxx}
Glycine	0.864	0.290	1.115	1.161
GABA	0.880	0.531	1.251	0.472

a Diazepam concentration = 1.6×10^{-4}M.

b Lecithin concentration = 15.542 ppm, cholesterol concentration = 1.175×10^{-6}M, Diazepam concentration = 0.75×10^{-4}M.

x Both permeants and diazepam in the compartment C and water in the compartment D.

xx Aqueous solution of diazepam in the compartment D and aqueous solution of permeants in the compartment C.

xxx Lecithin-cholesterol-diazepam mixture in compartment C together with the permeants and water in the compartment D.

xxxx Aqueous solution of lecithin-cholesterol-diazepam mixture in compartment D and permeant in compartment C.

will present a hydrophobic surface to the permeant. The data in Table V reveal that in the both orientations the diazepam liquid membrane impedes the transport of amino-acids.

The values of (ω/ω^{o}) in presence of lecithin-cholesterol-diazepam mixtures (Table V) appear to be interesting from the point of view of the drug action. When lecithin-cholesterol-diazepam are all present in compartment C (Fig. 1) along with the permeants the liquid membrane generated by lecithin-cholesterol-diazepam mixture which we shall call the composite liquid membrane, presents a hydrophilic surface to the permeants. Similarly when the permeants are present in the compartment C and the lecithin-cholesterol-diazepam mixture in the compartment D, the composite liquid membrane presents a hydrophobic surface to the permeants. In the former case the permeability of both glycine and GABA is enhanced considerably in comparison to the permeability values for blank experiments where no diazepam was used. The observation of increased permeability of GABA appears to have biological relevance

because in case of benzodiazepines biochemical[25,26] and neurophysiological[27-29] evidence has suggested that the antianxiety action of diazepam may be exerted by facilitating synaptic action of GABA in brain. Displacement of an endogeneous modulator protein forming part of a macromolecular complex constituting GABA receptor ionophores has been suggested[30] as one of the mechanism of such an action. However events at cellular and molecular level resulting in GABA potentiation are completely unknown[31]. The increased permeability of GABA through the lecithin-cholesterol-diazepam composite liquid membrane in the specific orientation of hydrophilic ends facing the permeants, as indicated in the present experiments can also be an explanation for facilitation of GABA action leading to antianxiety action of diazepam. Benzodiazepines are also known[32] to bind to sites that have high affinity for strychnine which is an antagonist of glycine. It has been suggested therefore that some actions of benzodiazepines may result from their interaction with glycine receptor[33]. Hence the observed increase in the permeability of glycine when it faces the hydrophilic surface of

the composite liquid membrane generated by the lecithin-cholesterol-diazepam mixture appears relevant.

The decrease in permeability of GABA (Table V) when it faces the hydrophobic end of the composite liquid membrane generated by lecithin-cholesterol-diazepam mixture does not appear to be relevant to the antianxiety action of diazepam. Nevertheless it can offer clue to the causation of certain other effects of diazepam. Diazepam has been shown to inhibit the uptake of GABA into synaptosomes prepared from mouse brain[34] and also in rat cortical slices[35]. Calcium dependent release of GABA has also been shown to be inhibited by diazepam[34]. The observed decrease in permeability of GABA when it preferentially faces the hydrophobic end of the lecithin-cholesterol-diazepam composite liquid membrane may be helpful in explaining these effects.

Thus the ability of diazepam to get incorporated in the liquid membrane generated by lecithin-cholesterol mixtures and modify the permeability of inhibitory-neurotransmitter amino acid molecules-glycine and GABA, appears to have a contribution to its actions.

STUDIES ON STEROIDAL DRUGS

Steroids are known to be surface active[36] and their interaction with constituents of biological membranes is also documented in literature[37]. In this section are reported studies on three representative steroidal drugs namely testosterone propionate - an androgen, ethinylestradiol - an estrogen and hydrocortisone acetate - a glucocorticoid. In these experiments also a cellulose acetate microfiltration membrane/aqueous interface has been chosen as site for the formation of the drug liquid membranes to highlight the role of passive transport through the liquid membrane in the mechanism of their action.

Data have been obtained to demonstrate the formation of liquid membranes by the steroidal drugs at the interface and also their incorporation in the liquid membrane generated by lecithin. Data have also been obtained on the transport of glucose and amino acids in presence of liquid membranes generated by the steroidal drugs and the mixture of lecithin and steroidal drugs to gain information on the role of liquid membrane formation in their action.

Materials and Methods:

Testosterone propionate (Organon (India) Ltd), Ethinylestradiol (Organon (India) Ltd.), Hydrocortisone acetate (Wyeth Laboratories Ltd.), Lecithin (Patel Chest Institute, Delhi), glucose, leucine, histidine, tryptophan (BDH) and distilled water, distilled once over potassium permanganate in all-pyrex glass still were used in the present experiments.

The critical micelle concentrations of the drugs as determined from the variation of surface tension with concentration are recorded in Table VI. Aqueous solutions of the the drugs and lecithin were prepared by adding the desired volume of their ethanolic solutions of known concentration to aqueous phase with constant stirring. In the aqueous solutions thus prepared the final concentration of ethanol was not allowed to exceed 0.1% by volume because it was shown by a control experiment that a 0.1% solution of ethanol in water, by volume, did not lower the surface tension of water to any measurable extent.

Table VI CMC values of the steroidal drugs.

Drug	CMC x 10^6 M
Hydrocortisone Acetate	4.50
Testosterone Propionate	3.87
Ethinylestradiol	0.27

The hydraulic permeability measurements in presence of various concentrations of the drugs - below and above the CMC, and the solute permeability measurements for glucose and three amino acids in presence of the liquid membrane generated by the drugs in both the orientations - the hydrophobic end facing the permeants and the hydrophilic ends facing the permeants - were made using the method described in the earlier section (pages 80-86 and 101-103). In the solute permeability measurements the concentrations of the steroidal drugs taken was always higher than their CMCs to ensure complete coverage of the supporting membrane with the liquid membrane. Hydraulic permeability data were also obtained at various concentrations of the drugs in presence of lecithin solution of fixed concentration. For this, solutions of various concentrations of the drugs preapred in an aqueous solution of lecithin of concentration 15.542 ppm were filled in the compartment C of the transport cell (Fig. 1) and the compartment D was filled with distilled water. This particular concentration of lecithin was chosen because it has been shown in an earlier

study[38] that at this concentration the liquid membrane generated by lecithin completely covers the supporting membrane. The solute permeability measurements were also carried in presence of the mixture of lecithin and the drugs. The composition of the mixture was such that the liquid membrane generated by lecithin completely covers the supporting membrane and was saturated with the drugs. The concentrations at which the lecithin liquid membrane would be saturated with the drugs were obtained from the hydraulic permeability data in presence of mixtures of lecithin and steroidal drugs. The concentration of lecithin in these experiments was kept fixed at 15.542 ppm and the concentration of testosterone propionate, ethinylestradiol and hydrocortisone acetate were 5.806×10^{-6}M, 3.374×10^{-7}M and 6.0×10^{-6}M respectively.

The amounts of glucose transported to the compartment D was estimated by spectrophotometric determination of its reaction product with dinitrosalicylic acid (DNSA)[38]. The amounts of amino acids namely leucine, histidine or tryptophan transported

to the other compartment were estimated by spectrophotometric determination of their reaction products with ninhydrin[24]. A Bausch and Lomb Spectronic - 20 Spectrophotometer was used for the measurements.

All experiments were done at 37 ± 0.1°C.

Results and Discussion:

The hydraulic permeability data in presence of various concentrations of the drugs were found to obey the linear relationship (2). The values of L_p were found to decrease with increase in the concentration of drugs up to their CMCs beyond which they become more or less constant. The values of (L_p/L_p^o) in one particular case of hydrocortisone acetate are plotted against concentration in Fig. 6. The trend in Fig. 6 is in keeping with Kesting's hypothesis[6-8] and is indicative of the liquid membrane formation. The values of Lp in case of hydrocortisone acetate computed, using mosaic membrane model[9,10], at concentrations lower than the CMC compare favourably with the experimentally determined values (Table VII) confirming the formation

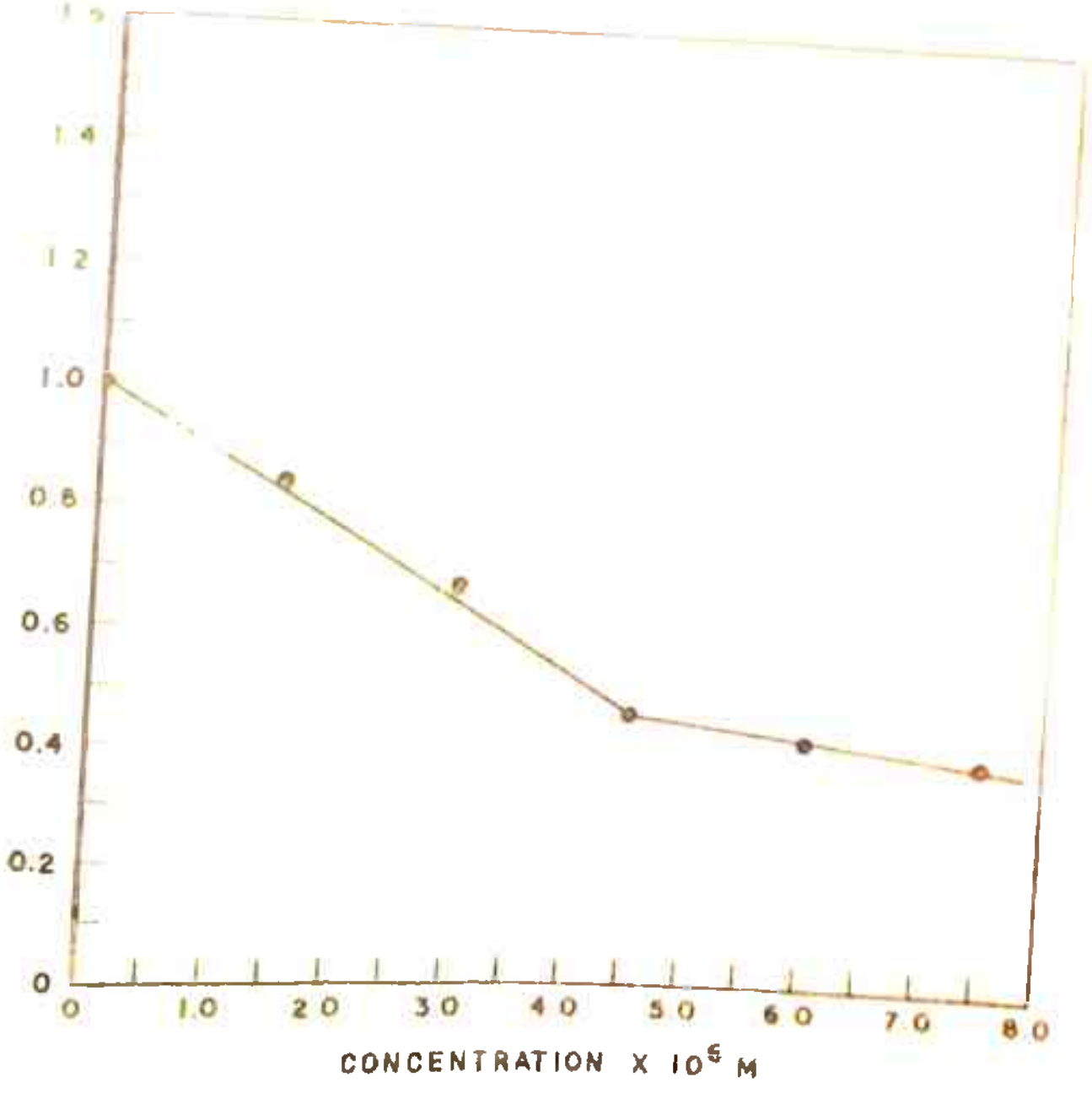

Fig. 6. Variation of Lp/Lp^o with concentration of Hydrocortisone acetate.

Table VII Values of L_p at various concentrations of hydrocortisone acetate.

Concentration x 10^6M	0.0	1.50 (0.33CMC)	3.00 (0.67CMC)	4.50 (1CMC)	6.00	7.50
L_p^{*} x 10^8($m^3s^{-1}N^{-1}$)	4.063 ±0.073	3.412 ±0.178	2.720 ±0.131	1.869 ±0.129	1.702 ±0.073	1.597 ±0.236
L_p^{**} x 10^8($m^3s^{-1}N^{-1}$)	-	3.338 ±0.092	2.595 ±0.107	-	-	-

x Experimental values

xx Computed values using mosaic membrane model.

of liquid membrane. Similar trends were observed in the case of ethinylestradiol and testosterone propionate also furnishing evidence in favour of liquid membrane formation.

The hydraulic permeability data at various concentrations of the steroidal drugs in presence of lecithin solution of fixed concentration 15.542 ppm, were also found to be in accordance with the linear relationship (2). The data in one particular case - in the case of hydrocortisone acetate in presence of lecithin are plotted in Fig. 7. The values of L_p/L_p, show a decrease with increasing concentration of hydrocortisone acetate upto 4.50×10^{-6}M beyond which they become more or less constant(Fig. 7). This indicates that hydrocortisone acetate gets incorporated within the liquid membrane generated by lecithin and when its concentrations equals 4.50×10^{-6}M, the lecithin liquid membrane is saturated with the drug. Similar trends were observed with ethinylestradiol and testosterone propionate also. The concentrations at which ethinylestradiol and testosterone propionate

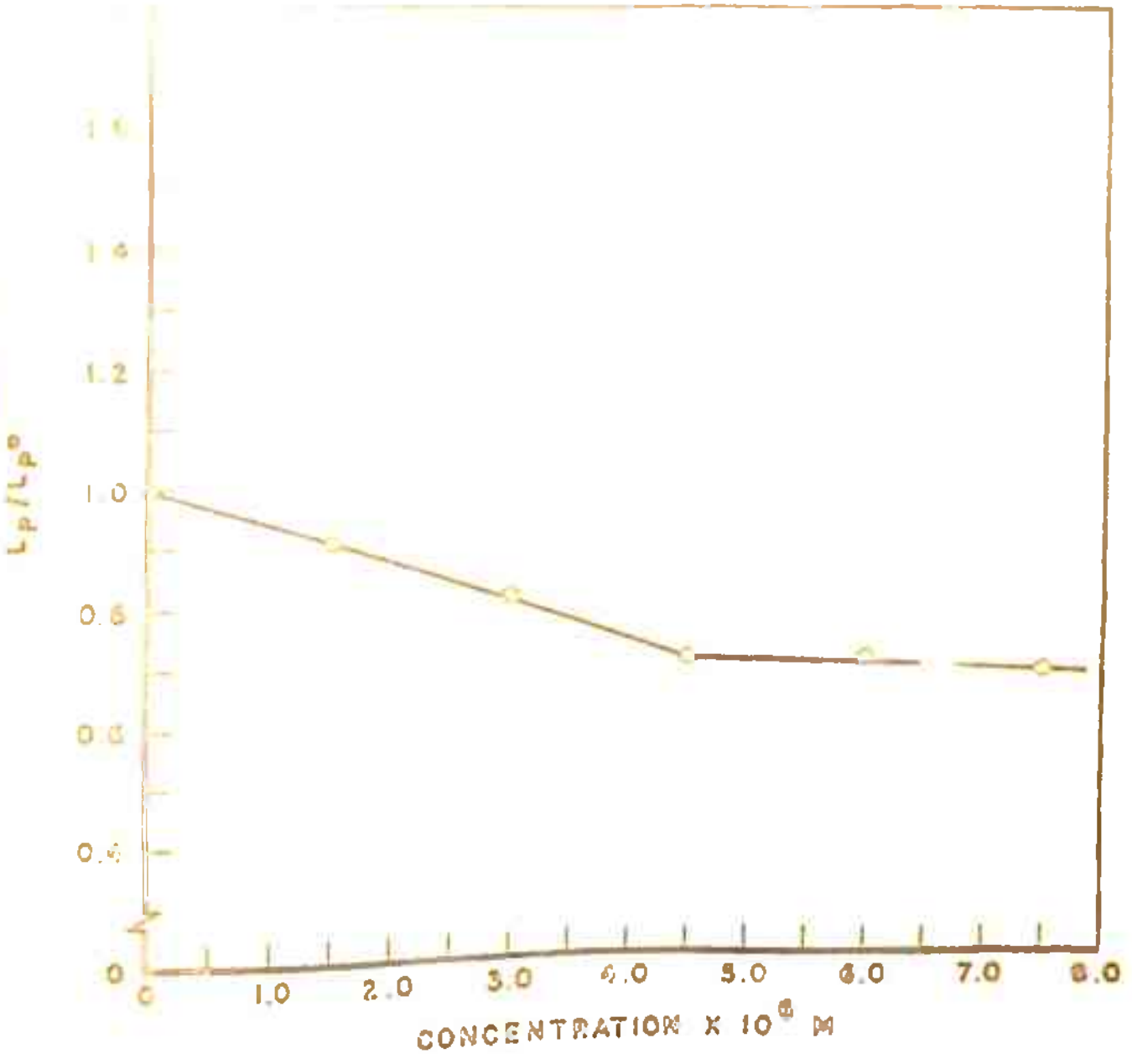

Fig. 7. Variation of L_p/L_p^o with concentration of Hydrocortisone acetate, in presence of Lecithin solution of fixed composition-15.542 ppm.

saturated the lecithin liquid membrane were found to be 2.699×10^{-7}M and 3.871×10^{-6}M respectively.

In order to ascertain whether the steroidal drugs which are incorporated in the lecithin liquid membrane reach the interface or not, surface tensions of solutions of various concentrations of the drugs prepared in aqueous solutions of lecithin of fixed concentration - 15.542 ppm, were measured. Out of the three drugs, only ethinylestradiol showed further decrease in surface tension of aqueous solution of the lecithin while the other two did not implying that only ethinylestradiol is found at the interface. This is in keeping with the fact that ethinylestradiol is more surface active than testosterone propionate and hydrocortisone acetate as is evident from the CMC values (Table VI).

The normalised values of the solute permeabilities (ω/ω°) for both the orientation of the liquid membrane - hydrophobic surface of the liquid membrane facing the permeants and hydrophilic surface of the liquid membrane facing the permeants, are recorded in Table VIII. The data in Table VIII

Table VIII Values of normalised permeability $r = (\omega/\omega^{o})$ of glucose, leucine, histidine, tryptophan in presence of steroidal drugs and lecithin-steroidal drug mixtures[a].

Permeants		Steroidal drugs		
		Testosterone propionate	Ethinyl-estradiol	Hydrocorti-sone acetate
Glucose	r^{b}	1.639	1.078	1.290
	r^{c}	1.650	1.224	1.434
	r^{d}	1.185	1.081	1.022
	r^{e}	1.073	1.144	1.206
Leucine	r^{b}	1.171	1.373	1.210
	r^{c}	0.538	0.585	0.619
	r^{d}	1.178	1.162	1.095
	r^{e}	0.990	0.980	0.752
Histidine	r^{b}	1.226	1.855	1.222
	r^{c}	0.545	0.778	0.490
	r^{d}	1.274	1.200	1.197
	r^{e}	0.930	0.773	0.718

contd.....

Table VIII contd.

Permeants		Steroidal drugs		
		Testosterone propionate	Ethinyl-estradiol	Hydrocorti-sone acetate
Tryptophan	r^b	1.337	1.448	1.160
	r^c	0.675	0.611	0.629
	r^d	1.400	1.258	1.143
	r^e	1.000	0.691	0.681

a Lecithin concentration in the mixtures 15.542 ppm.

b Permeants and the steroidal drugs in compartment C and water in compartment D.

c Permeants in compartment C and the steroidal drugs in compartment D.

d Lecithin-steroidal drug mixture and the permeants in compartment C and water in compartment D.

e Permeants in compartment C and lecithin-steroidal drug mixture in compartment D.

reveal the following trends. The permeability of glucose is enhanced in both the orientation of the liquid membranes generated by the steroidal drugs or lecithin steroidal drugs mixture. The permeability of amino acids, however, is enhanced only in the specific orientation of the liquid membranes with their hydrophilic surface facing the permeants. The cause for the enhancement of permeabilities is difficult to locate at this stage. Micelles playing a role in the transport, has been indicated in literature[39]. Although in the present experiments also micelles in the liquid membrane generating species were present in the bulk of the solution, because the concentrations chosen in the solute permeability experiments were higher than the CMCs, the role played by the micelles is difficult to pinpoint. Nevertheless, trends observed in permeability of amino acids (TableVIII) appear relevant to some of the biological actions of the steroidal drugs.

Permeability of the amino acids in presence of lecithin-steroidal drug mixtures is enhanced more in case of testosterone than ethinylestradiol. On

the contrary, in the absence of lecithin, the trend in permeability of amino acids was just the opposite (Table VIII). Since in biological cells androgens are known to be more anabolic than estrogens[40], incorporation of the steroidal drugs in the membrane phospholipids in the specific orientation of hydrophilic ends facing the approaching amino acids may be necessary for their anabolic action.

Glucocorticoids like hydrocortisone are known to mobilise amino acids from a number of tissues[41]. In the present investigation also it was observed (Table VIII) that hydrocortisone either by itself or in association with lecithin enhanced permeability of the amino acids. Since for this action, the specific orientation of the steroids with their hydrophilic ends facing the permeants was observed to be necessary (Table VIII), it is tempting to suggest that a similar orientation of glucocorticoids, like hydrocortisone may be necessary even in biological cells.

Unlike the effect on amino acids the uptake of glucose is reduced in the presence of glucocorticoids[41]. The present experiments, however, showed

an increase in permeability of glucose in presence of the steroids in both the orientations - the hydrophilic ends facing the permeants and the hydrophobic ends facing the permeants. Hence the present observations on the increase in permeability of glucose 9(Table VIII) do not appear to be relevant to the biological effects of glucocorticoids on glucose transport.

Steroids are known to exert their anabolic action by combining with a soluble receptor present in cytoplasm[42] of the target cells. Since these actions are accompanied by increased mobilisation of amino acids and synthesis of proteins the liquid membranes generated by the steroidal drugs in association with phospholipids like lecithin may have a role to play in the mechanism of their action.

STUDIES ON PROPRANOLOL

In this section are reported studies on propranolol which is a representative β-blocker drug. Incidently, not only surface activity of β-blockers is documented in literature[43,44] but

biological effects of β-blockers like effect on myocardial conduction velocity have been shown to correlate[45] with their surface activity and hydrophobicity. It is further indicated[46] while studying the effects of drugs like reserpine, chlorpromazine, imipramine and propranolol, that irrespective of chemical structure, the surface activity of these drugs mainly determines their potency to affect all kinds of membranes, especially that of catecholamine storing particles.

Data have been obtained to demonstrate the existence of a liquid membrane, generated by propranolol, at a cellulose nitrate micrdfiltration membrane/aqueous interface. Data on transport of adrenaline through the liquid membrane thus generated by propranolol in series with a supporting membrane, have been obtained and discussed in the light of mechanism of action of drugs. The data indicate that the liquid membrane generated by propranolol may contribute significantly to the mechanism of its action.

Materials and Methods:

Propranolol hydrochloride (Sigma Chemical Co. USA), Adrenaline acid tartrate (Loba Chemie) and distilled water, distilled once over potassium permanganate in all-pyrex glass still were used in these experiments.

The CMC of aqueous propranolol was found to be 4.75×10^{-5}M. The hydraulic permeability data in presence of various concentrations of propranolol utilised to demonstrate the liquid membrane formation were obtained using the method already described (section dealing with local anaesthetic). The solute permeability (ω) data for adrenaline for the two orientation of the drug liquid membrane - hydrophilic ends and hydrophobic ends of the propranolol liquid membrane respectively facing the permeant, were obtained using the method described in sections dealing with local anaesthetics, diazepam and steroidal drugs.

Since propranolol was observed to interfere with fluorimetric estimation of adrenaline,

the amount of adrenaline transported to the compartment D was measured spectrophotometrically. For this a calibration curve was constructed by noting the absorbance of solutions of varying concentrations of adrenaline prepared in a solution of fixed concentrations of propranolol, which was equal to the concentration of propranolol used in solute permeability experiments. The calibration curve was found to obey Beer's law. A Cary 17-D spectrophotometer was used for absorbance measurements at 285 nm - the absorption maxima for adrenaline.

All measurements were carried out at 37 ± 0.1°C.

Results and Discussion:

The hydraulic permeability data at various concentrations of propranolol were found to be in accordance with equation (2). The normalised values of L_p - the values of (L_p/L_p^o) where L_p^o is the value of L_p when propranolol concentration was zero, are plotted against propranolol concentration in Fig. 8. The values show a progressive decrease with increase in concentration of propranolol upto its CMC where after they become more

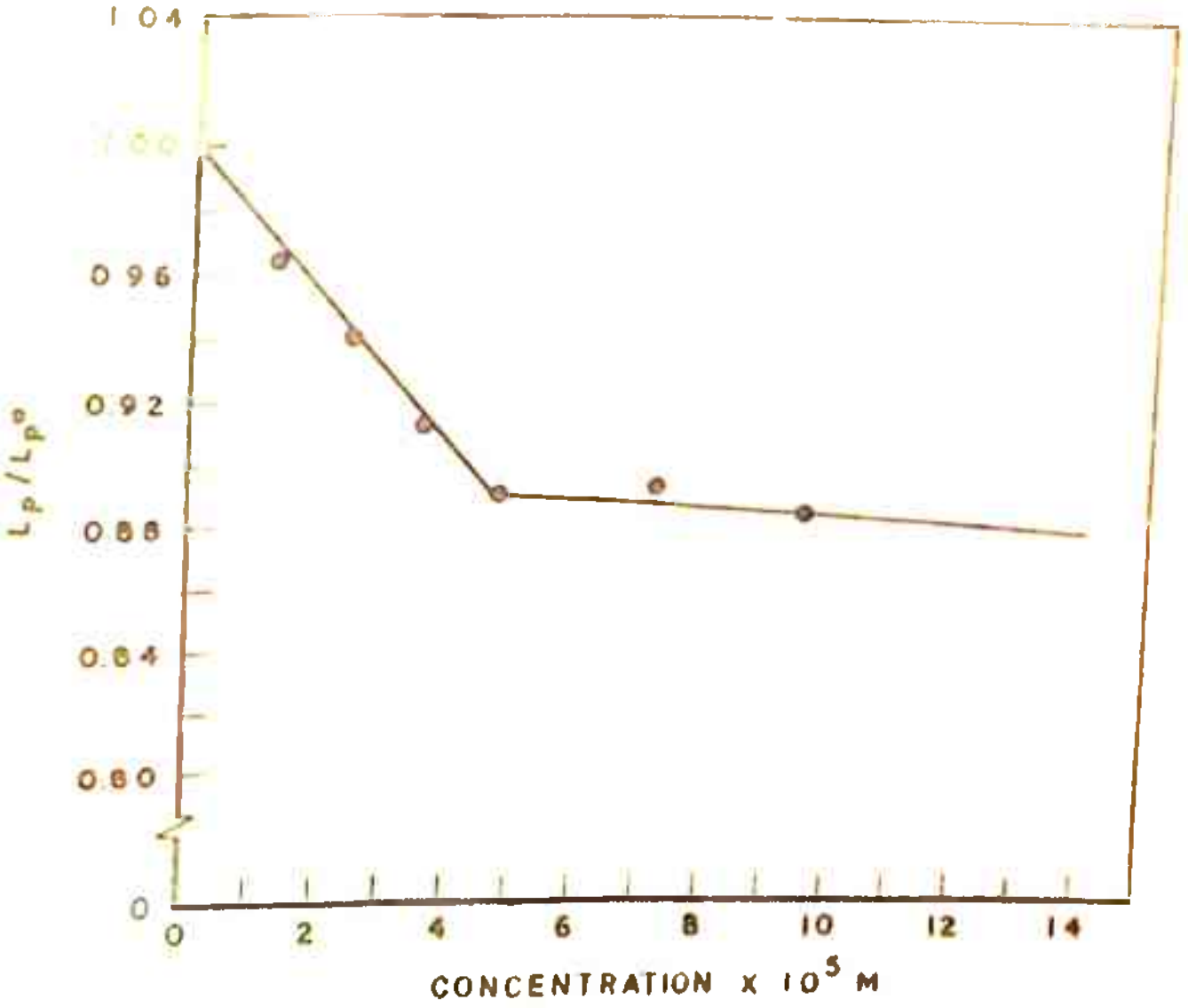

Fig. 8. Variation of L_p/L_p^o with concentrations of Propranolol Hydrochloride.

or less constant. This trend is in keeping with the liquid membrane hypothesis[6-8] according to which as concentration of the surfactant is increased, the interface gets progressively covered with the surfactant layer liquid membrane and at the CMC it is completely covered. The marginal decrease beyond the CMC may be due to densing of the liquid membrane as postulated in the liquid membrane hypothesis.

The normalised values of solute permeability (ω/ω^{o}) for adrenaline for both the orientation of the propranolol liquid membrane, are recorded in Table IX. Although permeability of adrenaline is reduced in both the orientations of the liquid membrane, the reduction in permeability is maximum when the permeating adrenaline molecules face the hydrophobic surface of the liquid membrane generated by propranolol (Table IX).

Since propranolol is known[47] to act by antagonising actions of catecholamines, the specific orientation of the propranolol molecules with their hydrophobic ends facing the permeant - adrenaline - may be necessary even in biological cells. This

Table IX Values of normalised permeabilities (r) of adrenaline acid tartrate in presence of propranolol hydrochloride.

	r^{x}	r^{xx}
Adrenaline (acid tartrate)	0.787	0.345

Propranolol hydrochloride concentration = 7.317×10^{-5}M.

x Propranolol hydrochloride is in compartment C along with permeant.

xx Propranolol hydrochloride is in compartment D and permeant in compartment C.

implies that the orientation of β-receptor should be such that their hydrophilic groups get attached to the hydrophilic groups of catecholamines or their antagonists leaving their hydrophobic groups projected outwards to face the permeants. The phenolic hydroxy groups have been indicated to be of particular relevance to the intrinsic activity of β-receptors[43].

Propranolol is known to be one of the highly potent, non-selective β-adrenergic blocking agent[47]. Inhibiting action of adrenaline as a result of β-blockage has been shown to prevent local edema formation in response to the infection of a variety of irritants in rat paw[49]. Propranolol has been shown to block antianaphylactic effect of catecholamines on antigen-induced histamine release from sensitized lung[50]. Reduction in circulating eosinophils, characteristically induced by adrenaline is blocked by propranolol[51]. Propranolol has also been shown to inhibit uptake and release of catecholamines[46].

Although majority of these effects are due to reduction in permeability of catecholamines, which is by active mechanism[52], the present data (Table IX) indicate that the liquid membrane generated by propranolol also contributes to the mechanism of its action by offering resistance to transport of adrenaline. Although impediment in the transport of adrenaline, as observed in the present experiments (Table IX) is passive in nature, it is likely to be accompanied by reduction in its active transport as well. This is because access of the permeable-substances to the active site located on biomembranes is likely to be reduced due to the propranolol liquid membrane interposed in between.

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CHAPTER 5

CONCLUDING REMARKS

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The experiments on photo-osmosis described in Chapter 2 indicate that the liquid membrane bilayers generated using Kesting's hypothesis, can also be used as model system for biomembranes. Nevertheless there is a need to mimic many more biologically relevant transport processes - active and facilitated processes on such liquid membrane bilayers to establish the workability of such liquid membrane bilayers as model systems for biomembranes. The work on these lines is tempting because the liquid membrane bilayers appear to have one advantage over BLMs. In BLMs the lipid molecules are more tightly packed while in biomembranes the lipid bilayer are in a fluid state. The liquid membrane bilayers generated using Kesting's hypothesis are expected to have more fluidity in them and hence in this respect are expected to be closer to the state of lipid bilayer in biomembranes. However much more work

on the characterisation of the liquid membrane bilayers is necessary to establish their credentials as model system for biomembranes.

The studies reported on drug action lend credence to the surmise that all surface active drugs may have a common mode of action. The liquid membrane generated by the surface active drugs at the site of action of the respective drugs acting as a barrier for the transport of relevant permeant appears to be an important step common to the mechanism of action of all surface active drugs. This is a new facet of drug action which had hitherto gone unnoticed because passive transport has been traditionally considered unimportant for drug action. This by no means should imply that the active interaction of drugs with receptors is unimportant. But nevertheless, the liquid membrane aspect of drug action fills an important knowledge gap and deserves incorporation in the theories of drug action. This appears to be an interesting line of future work.

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6. WCR C541 Environmental Chemistry.
7. WCR C542 Enzyme Kinetics.
8. GER N101T Beginning German.
9. GER N102T Technical German.
10. BITS C791T Teaching Practice I
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