STUDIES ON
LIQUID MEMBRANE PHENOMENON
IN BIOLOGICAL ACTION

A Thesis

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By

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TO ALL MY TEACHERS
WHO TAUGHT ME FROM
ALPHABET TO THE
LIQUID MEMBRANE PHENOMENON

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI, RAJASTHAN.

CERTIFICATE

This is to certify that the thesis entitled
"STUDIES ON LIQUID MEMBRANE PHENOMENON IN BIOLOGICAL
ACTION" submitted by Mr. Anantha Naik Nagappa,
ID.No. 85P2X7401, for the award of the Ph.D. degree
of the Institute, embodies original work done by him
under my supervision.

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ANANTHA NAIK NAGAPPA

PREFACE

Liquid membrane hypothesis for the action of surface active biological agents has been proposed recently (Adv. Colloid Interface Sci., 20, (1984) 131-161). The central concept in the hypothesis is that surface active biological agents may generate a liquid membrane at the site of action, which acts as a barrier modifying transport of relevant molecules to these sites ---- this of course is in addition to the concepts like structural complimentarity of the antagonists enabling them to interact with the same receptor sites with which the agonist molecules interact. The liquid membrane generated by the biological agent/ drug itself contributing to its biological action is a new facet of the mechanisms of biological actions which had hitherto gone unnoticed. The liquid membrane hypothesis for biological action, when viewed in the light of existing theories, leads to a more rational biophysical explanation of such agents which act by modifying the permeability of cell membrane. Although several studies substaintiating the liquid membrane hypothesis have been conducted in past few years, there is a need to investigate many more biological agents for

the role of liquid membrane phenomenon in their action. This need is quite pressing because the liquid membrane hypothesis of biological actions is of very recent origin.

In the present thesis, therefore, studies on the following categories of biological agents are reported: (i) gonadal steroid harmones, (ii) prostaglandins, (iii) anticancer drugs (5-fluorouracil and its derivatives and (iv) vitamin-E (α -tocopherol). The thesis is divided into seven Chapters. Chapter I is concerned with liquid membrane hypothesis of biological action and its implications. Chapter II gives a generalised version of the experiments carried out. Chapters III to VI give an account of authors own contributions, experimental investigations carried out by the author on the four categories of biological agents listed above. Chapter VII is the summary of the contents of the thesis.

ANANTHA NAIK NAGAPPA

CHAPTER 1

THE LIQUID MEMBRANE HYPOTHESIS - BIOLOGICAL
IMPLICATIONS

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Surface active molecules when added to aqueous
phase in contact with 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 concentra-
tion (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 interface 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 (PVM) to saline feed in
reverse osmosis dramatically enhances the salt reten-
tion capacity of cellulose acetate membranes with but
a small decrease in the flux of product water, was
explained by Kesting[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 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 by the surfactant layer liquid membrane and at the CMC it is completely covered. Srivastava and associate, using osmosis and electroosmosis as probes have further substantiated the liquid membrane hypothesis.[6-8]

Since molecules of surface active nature are crucial to living matter and its organisation[9], the hypothesis[3-5] that the surfactant layer generated at the interface acts as a liquid membrane can have significant biological implications.

BIOLOGICAL IMPLICATIONS

Model Systems for Biomembranes

Every organism unicellular or multicellular communicates with its environment. The instruments of communication are embedded in the membranes 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 investigation on these much simpler model systems will provide insights for understanding similar and comparable phenomena in biological membranes. Biological membranes are visualised[10] 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 systems for this core, the bilayer lipid membrane or the black lipid membrane (BLM) as it is also sometimes called, developed by Mueller, Rudin, Tien and Wescott[11-16] is the model system which has been most widely experimented with. Tien's excellent monograph[16] contains a comprehensive account of theoretical and practical aspects of BLM. It has been successfully demonstrated[15,16] that biologically relevant transport processes can be mimicked on BLMs after incorporating relevant protiens or other molecules in them. Although BLM, todate, 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[11,17-19] much higher
than those reported for biomembranes[20]. Also the rate
of passive ionic diffusion through BLMs is much slower[21,22]
than through biomembranes.[23-24]. This has been ascribed[25]
to a tight molecular arrangement of lipid molecules in
the BLMs white in biomembranes the lipid bilayer is
in a fluid state[10]. In addition BLMs are fragile struc-
tures and, hence difficult to work with. Realising
this, Tien and associates have recently developed[26,27]
an alternative method using a microporous supporting
membrane which improves the stability and surface area
of the BLMs. The new method as claimed by the authors[26,27]
yields _in Situ_ formation of a large number of micro
BLMs in the pores of the supporting membrane which have
a much longer life and better manipulability. Although
Tien and associates[26,27] have succeded in mimicking
a few biologically relevant processes on the new model
system, its complete characterization as has been done
in the case of traditional BLMs is still awaited.

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,
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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 lipids, one liquid
membrane on either side of a hydrophobic supporting
membrane. Can the liquid membrane bilayers thus generated
work as model systems for biomembranes ? Srivastava
and colleagues have explored this possibility in a
number of investigations[28-36]. In these studies bilayers
of liquid membrane have been generated from constituents
of biomembranes on a hydrophobic supporting membrane
and transport accross them have been studied. Not
only have the passive transport data on the liquid
membrane bilayers thus generated been shown closer to
the corresponding data on biomembranes[29]; some of the
biologically relevant transport processes have also been
mimicked on the liquid membrane bilayers, the notable
membrane mimetic experiments are on the light induced
transport processes e.g. photoosmosis and photoelectric
effects[31-32,34-36] and the transport characteristics of
the liquid membrane bilayers in the presence of polyene
antibiotics[33]. The trends in the data obtained from the
experiments on the liquid membrane bilayers were shown
to be consistent with the trends reported on BLMs. This
consistency established the credentials of the liquid
membrane bilayers as model system for biomembranes
Nevertheless there is a need to mimic many more biologi-
cally relevant transport processes e.g. antigen/antibody
reactions, active transport processes etc to, further

establish the workability of the liquid membrane
bilayers as model systems for biomembranes.

The other important biological implication that
has been investigated by Srivastava and his group is
the role of the phenomenon of liquid membrane formation
in the mechanism of action of drugs which act by
altering the permeability of cell membranes. It is
discussed in the next section.

Drug Action

Formation of cell membrane and location of
receptor proteins in the lipid bilayer part of the biomembrane
is a consequence of surface activity. It is, therefore, logical
to expect that the drugs acting by modifying the permeability
of cell membranes after interacting with them are also
surface active in nature. A wide variety of drugs are
in fact surface active in nature[37-43]. In many cases
excellent correlations between surface activity and
biological effects have been demonstrated[44-50]. While
investigating the actions of drugs like reserpine,
prenylamine, chlorpromazine, propranolol, etc., it has
been concluded[51] that irrespective of chemical structure,
the surface activity of psychotropic drugs mainly deter-
mines 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[52], 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 action of all surface active drugs. Investiga-
tions carried out by Srivastava and associates, on a
wide variety of drugs have substantiated this surmise[53-66].
These investigations have lead to what can be called
as a "liquid membrane hypothesis of drug action".
A consolidated account of this hypothesis and its
discussion in the light of existing theories of drug
actions particularly occupancy theory [67,68] and the
rate theory[69,70] is contained in the article by Srivastava,
Bhise and Mathur.[71] Before we present a summarized account
of the "Liquid Membrane Hypothesis of Drug Action" a
summarized account of the "Occupancy theory"and the
"Rate theory" is in order.

Occupancy Theory :

Biologic responses to drugs are, as a rule, graded; they can be measured on a continuous scale. There is a systematic relationship between the dose of a drug and magnitude of the response. Application of law of mass action to the dose response relationship was largely by Clark[67,72]. An observed biological effect was assumed to be a reflection of the combination of drug molecules with receptors. The magnitude of a response was postulated to be directly proportional to the occupancy of receptors by drug molecules. The maximal response is assumed to be obtained when all the receptor are occupied.

Simple mass law principles enable to express quantitatively the dependance of biological effect upon dose;

if $[X]$ represents concentration of the drug at the site,

$[R]$ represents concentration of receptors, not occupied by the drug,

$[RX]$ represents concentration of drug-receptor complex, and

$\triangle$ represents magnitude of biological response,

then for the equillibrium,

$$R + X \underset{K_2}{\overset{K_1}{\rightleftharpoons}} RX$$

One can write $\dfrac{[R][X]}{[RX]} = \dfrac{K_2}{K_1} = K_x$ .... (1)

Further, since biological response is assumed to be proportional to the concentration of occupied receptors, we can write,

$$\triangle = K_3 \, [RX] \qquad \qquad ...(2)$$

In equations (1) and (2) K_1, K_2, K_3 are corresponding rate constants and K_x is the dissociation constant of drug receptor complex.

The total receptor concentration R_T is given by Equation.

$$[R_T] = [R] + [RX] \qquad \qquad ... (3)$$

Substituting the value of $[R]$ from equation (3) equation (1) becomes

$$\frac{\left\{ [R_T] - [RX] \right\} [X]}{[RX]} = K_x \qquad \qquad ... (4)$$

which after rearrangement can be written as

$$\frac{[RX]}{[R_T]} = \frac{[X]}{K_x + [X]} \qquad \qquad ... (5)$$

In view of the fact that maximum biological response, which the system is capable of, is obtained only when all the receptors are occupied i.e. $\triangle_{max} = K_3 \, [R_T]$,

the equation (5) can be rewritten as

$$\frac{\Delta}{\Delta_{max}} = \frac{[RX]}{[RT]} = \frac{[X]}{K_x + [X]} \qquad \dots \quad (6)$$

It has been pointed out[73] that the equation (6) has in it the following implicit assumptions.

(1) An all-or-none stimulus is elicited by the combination of each receptor site with an agonist molecule.

(2) There is summation of these individual stimuli;

(3) The effect is linearly proportional to the number of stimuli;

(4) The maximal stimulus occurs when every receptor site is occupied by an agonist molecule.

(5) The drug receptor complex is formed by readily and rapidly reversible chemical bonds.

(6) The occupation of one receptor does not affect the tendency of other receptors to be occupied.

Although explanation of observations related to the response caused by most agonist molecules can be provided on the basis of occupancy theory, the observations related to responses caused by a variety of

other agonist molecules need postulation of a few
additional concepts which are summarised below.

Affinity:-

If a set of LDR (Log dose response) curves for
a series of congeneric drugs of varying potencies
interacting with the same receptor are examined, it
is observed that these curves do not overlap. These
LDR curves indicate that for a particular biological
response to be elicited, the most potent agonist drug
requires the least concentration. This is expressed
by saying that the most potent drug has highest affinity
for the receptors while the congeners have lesser
affinity. From equation (5) which can be rearranged
to read,

$$\frac{[RX]}{[R_T]} = \frac{1}{1 + \frac{K_X}{[X]}} \qquad \ldots(7)$$

it follows that[74] the ratio $\frac{[RX]}{[R_T]}$ increases with concen-
tration of the drug $[X]$ and decreases with dissociation
constant Kx of the drug-receptor complex $[RX]$. Thus
"affinity" of the drugs to the receptors is proportional
to the reciprocal of Kx.

Thus more is the potency of an agonist, higher will be its affinity for the receptor and hence lower will be the dose required to elicit a particular quantum of biological response.

If LDR (Log Dose Response) curves for an agonist alone and for a mixture of agonist and competitive antagonist are compared, it can be inferred that LDR for the mixture shows a behaviour similar to that of the agonist but with lesser affinity for the receptor. This indicates that presence of a competitive antagonist alters the effective affinity of the agonist for the receptor.

Efficacy (Intrinsic Activity):

According to the occupancy assumption the magnitude of a response is determined by the number of receptors occupied. Agonist drugs are supposed to differ in their affinity for the receptors and therefore different doses are required to achieve the same degree of receptor occupancy and hence the same response. A molecule of any agonist occupying a given receptor site is assumed to make the same quantal contribution to the over all response as a molecule of any other agonist. It may, however, occur at a higher concentration

of the other agonist if its affinity is low.

Instances are known in which various agonists that apparently act on the same receptor site produce maximal responses of different magnitudes, an observation not accounted for by the theory. Hence the theory has been modified by introducing the concept of intrinsic activity[75] or efficacy[76]. It is defined as capacity of a drug to initiate a response once it occupies the receptor sites.

Thus, affinity describes tendency of the drug to form a stable complex with receptor and efficacy describes the biologic effectiveness of the drug-receptor complex. The two properties are considered to be unrelated. Since biological effect would be determined both by the extent of receptor occupancy i.e. affinity and also by efficacy, it follows that equal biological responses need not imply equal degree of receptor occupancy and maximal responses may vary from drug to drug.

According to Ariens[75], equation (6) should be rewritten as

$$\frac{\Delta}{\Delta_{max}} = \propto \frac{[RX]}{[R_T]} \qquad \ldots \quad (8)$$

where α is termed as intrinsic activity[75] factor. In Stephenson's alternative frame work[76], efficacy ($\bar{e}$) denotes capacity of a drug to initiate a response once it occupies receptor sites. The value of parameter "e" can range from zero to a large positive number.

In the sequence of events represented by the equation

$$X + R \xrightarrow[K_2]{K_1} RX \xrightarrow{k_3} \text{Response}$$
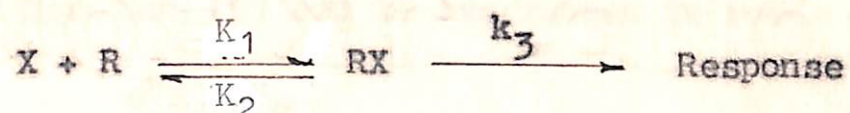
the rate constant k_3 is related to efficacy. It is viewed as a measure of the probability that an agonist occupying a receptor will induce a shift to the configuration that provides the stimulus.

Stimulus 's' and efficacy 'e' can be related by following equation

$$S = e\frac{[RX]}{[R_T]} = \frac{e[X]}{K_X + [X]} \qquad \cdots \qquad (9)$$

Thus as far as receptor occupation is concerned, stephenson's efficacy (e) and Arien's instrinsic activity factor (α) are the same. However the factors differ when the relationship between receptor occupancy and response is considered.

In Stephenson's framework biological response
is a function of the stimulus

i.e.,

$$\frac{\Delta}{\Delta_{max}} = f(s) = f\left(\frac{e\,[RX]}{[R_T]}\right) \qquad .. \quad (10)$$

The relationship between response and stimulus is
arbitrarily defined such that S = 1 when response is
half the maximal response produced by a highly active
agonist. Equation (9) can be rearranged to read

$$S = \frac{e\,[X]\,/K_x}{1 + [X]/K_x} \qquad ... \quad (11)$$

From equation (11) it follows that, for a highly active
agonist, where 'e' has a high value,

$$S \longrightarrow e\,[X]/K_x$$

Spare Receptors: [77,78]

According to the assumptions made in occupancy
theory, maximal response is attainable only when all the
receptors are occuped by the agonist drug. As a corollary;
therefore, when an antagonist is added to the system,
at no stage maximal response should be attainable. However,
in case of competitive antagonism, this prediction is not
observed to be true, i.e., even in the presence of a compe-
titive antagonist the same maximal response is attainable,

which is obtained in the absence of an antagonist, but
at a higher concentration of the agonist. This discrepancy
has been resolved by proposing spare receptors". It is
hypothesized that there are some additional receptors
which become available to the agonist in presence of an
antagonist. It is also stated that in obtaining maximal
response due to agonist alone, there is no combination
between agonist and the so called "spare receptors".

In short, for a highly active agonist with a
high efficacy the maximal response will be produced by
a concentration that does not occupy all the receptors.
These receptors which remain unoccupied are termed "spare
receptors".

It is also been suggested[79] that it is better
to hypothesize 'spare cells' rather than "spare receptors".
An antagonist being applied for a short time, would block
only the superficial cells and not deep ones. Experimental
evidence[80], in case of α-adrenergic receptors of rat
vasdeferens does not provide evidence for spare receptors.
Paton[69,70] has commented that for occupancy theory,
existence of spare receptors merely seems to be a puzzling
extravagance. The "spare receptors", thus continues to
be a hypothetical assumption.

Rate Theory: [69,70,81]

The central idea in this theory is different from that in the occupancy theory. Instead of attributing excitation to the occupation of receptors by drug molecules, it is attributed to the process of occupation each association between a drug molecule and a receptor providing one quantum of excitation. The magnitude of biological response is proportional to the rate at which drug molecules associate with receptor sites. This rate depends on the concentration of free drug, the concentration of free receptor sites and K_1, the rate constant for association of drug molecules with receptors.

This theory abandons the occupancy assumption and adopts the principle of intrinsic activity. Efficacy, in this theory, is no longer an adhoc constant but defined by the rate constant, K_1 which may differ from drug to drug. The distinction between an agonist and an antagonist is based on the value of K_2, the dissociation rate constant for drug receptor complex. Drugs with higher values of K_2 are agonists because if K_2 is large, the rate of dissociation of drug-receptor complex will be high making free receptor sites available at high rate for new effective collisions with drug molecules. In contrast, if K_2 is small, drug-receptor complex will be

more stable, the rate of dissociation will be low, making
the availability of free receptors to the drug molecules
for new association events infrequent. This will conse-
quently lead to little or no excitation. Thus, the drugs
with low K_2 will act as weak agonists or as antagonists.
Antagonism, therefore, implies persistent occupancy of
the receptor by the drug-antagonist. Both for agonists
and antagonists potency is determined by the equilibrium
dissociation constant K_2/K_1 which describes affinity of
the drug for receptor.

The theory explains why antagonists tend to be
bulkier than agonists since it is indicated that as compared
to small molecules, bulky molecules may have more non-
specific binding and hence a lower dissociation constant.
It is also claimed that the theory explains why potent
antagonists have a slow onset of action since more potent
they are, lower is the dose at which they must be used
and consequently slower will they equilibrate. Both these
arguments apply with equal force to the mass law theory
based on the occupancy assumption.

Rate theory has been used[69] to predict slopes
of LDR curves which are found to depend on association
and dissociation rate constants.

Since agonists have high dissociation rates than antagonists, postulating a large shift an antagonist occupancy during exposure to agonist for a short time appears unreasonable. Yet it is on such a postulate that a quantitative account of competitive antagonism rests, and it is generally accepted that the theory describes experimental results with considerable accuracy[70]. If it is accepted that agonists and antagonists combine with receptors in a mutually exclussive way, then it is expected that the extent of dissociation of the antagonist from the receptor taking place during the brief testing period with doses of agonist must be very small. Since a large response may be produced with in a few seconds of adding a dose of agonist, even while almost all the receptors are still occupied by antagonist indicates that agonist occupancy responsible for higher response can only be very limited. This argument directly leads to the spare receptor hypothesis, the only variation in arguments of rate theory is that the notion of spare receptors is replaced by that of spare capacity for more rapid association.

The Liquid Membrane Hypothesis for Drug Action[71]

The two theories summarized above, though differ in arguments, have a common premise that the observed biological effects are a consequence of interaction of

drugs with membrane components. The antagonistic drugs, in general, are stated to interact with the membrane components and occupy the sites with which the agonist drugs would have interacted to give the desired biological response. Thus it can be stated that antagonistic drugs act by creating hindrance in the interaction of agonist drugs with receptor sites. How is this hindrance created, is contained in the liquid membrane hypothesis for drug action, which has been substantiated through investigations on a variety of drugs belonging to different pharmacological categories[53-66].

The membranes represent an interface. As a corollary any drug which acts by modifying the permeability of cell membranes after interacting with them, of necessity, has to be surface active in nature. Since surface active substances are capable of forming liquid membranes which can influence mass transfer across the interface (Kesting's Hypothesis) the formation of liquid membrane at the site of action could be an important event in the mechanism of action of surface active drugs. Thus the central concept in the liquid membrane hypothesis for drug action is that surface active drugs may generate a liquid membrane at the site of action which act as a barrier modifying the transport of relevant molecules to these sites. This is in addition to the concepts like structural complimentarity of the antagonist drugs enabling them to interact

with the same receptor sites with which the agonist mole-
cules interact. The liquid membrane generated by the
drug itself, acting as barrier modifying access of relevant
molecules to the site of action is a new facet of drug
action. If this concept is viewed in the light of the
"occupancy theory" and the "rate theory", a more rational
biophysical explaination for the action of surface active
drugs acting by modifying the permeability of cell
membranes, emerges.

It may, however, be clarified that the liquid
membrane hypothesis in no way disputes the specific/
active interaction between the agonist drugs and their
receptors. The liquid membrane formation is an event
which precedes the active interaction. The new point
of the hypothesis lies in the assertion that the passive
transport which has traditionally been considered un-
important for biological action also makes significant
contributions-transport through the liquid membranes are
indeed passive in nature.

Implications of the Hypothesis:

The liquid membrane hypothesis can provide a
clue to their quantitative action. This is because CMC
of the drug indicates the concentration at which the

interface will be completely covered by the drug liquid membrane. At this concentration (CMC), therefore, modification in the permeability of biological membrane would be maximum. This implies that at the CMC, magnitude of biological effect would also be maximum. Hence lower the CMC of a drug, lower is the concentration required to alter the membrane transport and as a consequence, more potent would be the drug. Thus CMCs of a series of drugs with the same pharmacological action can be a good indicator of their potency. The investigations of haloperidol[53] and chlorpromazine[57] justify this conjecture. CMC for haloperidol is 1×10^{-6}M while that of chlorpromazine is 4.5×10^{-5}M. Haloperidol is known to be more potent than chlorpromazine on milligram basis.[82] Another example substantiating this result is that of local anaesthetic drugs: the lower the CMC the more potent the drug. In a series of local anaesthetics, it was found[58] that CMCs and minimum blocking concentrations (MBC) for nerves are identical. This indicates that formation of liquid membrane between cations like sodium, potassium and the nerve membrane appears to be an important step in the mechanism of action of local anaesthetics. It is proposed that interaction of local anaesthetics with the lipid micro-environment of the sodium channel results in its fluidisation causing blockade of sodium transport.[83] Thus a physical mechanism can provide satisfactory explanation for local anaesthesia. Formation of liquid membranes

by these drugs within sodium channels and polar head
interaction of the drugs with the liquid microenvironment
of the channels can, therefore, explain why nerve block-
ing concentrations and CMCs are identical.

The liquid membranes as generated by surface
active drugs are expected to have two types of orientations
with respect to the approaching permeants. The drug liquid
membrane can present either hydrophilic or hydrophobic
ends to the permeants. It is observed that a change in
orientation of the drugs can alter transport of permeants.
Whichever orientation shows alterations in permeability,
similar to those observed in biological cells, is of
predictive value. In the majority of drug investigated
so far, it was found that resistance to transport of
permeants is maximum when hydrophobic ends of the surface-
active drugs face the approaching permeants. This implies
that the receptors for these drugs are likely to be
oriented in such a manner that their hydrophilic moieties
are projected outwards to which hydrophilic ends of the
drugs get attached. Therefore the hydrophobic ends of
the drugs project outwards to face the permeants. Such
an orientation can be rationalised if one examines the
nature of receptors, in general, in relation to the
lipid bilayers part of the biomembranes.

The receptors, in general, are membrane proteins and hence should be surface active in nature. Hence they should have hydrophilic and hydrophobic moieties in their structures. Since the exterior environment of biological cells is aqueous in nature, it is logical to expect that hydrophobic part of these membrane proteins will be associated with hydrophobic core of the lipid bilayers and only hydrophilic part will face the exterior. Prediction about similar orientation of receptor proteins and also the membrane proteins, in general, has been made in literature[84]. Thus the studies on liquid membrane generated by drugs are capable of indicating the possible orientation of receptors, responsible for interaction with the drugs.

Since the biological membrane is comprised of different types of lipids and proteins, a drug can alter transport across the membrane by one of the following mechanisms:

(i)   the drug itself may form a liquid membrane which can reasonably explain alteration of transport across the membrane;

(ii)   the drug lipid interaction may be responsible for the observed biological effect; or

(iii) the drug protein interaction may be the causative
action.

In the case where first possibility is ruled
out, because an effect similar to that on biological
tissues is not mimicked by the drug liquid membrane
alone, interaction with the liquid membrane formed by
the lipids needs to be studied. In case of diazepam[56],
it was found that the biological actions of the drug i.e.
facilitating actions of GABA could not be mimicked in either
orientations of the drug, but interaction with lecithin
liquid membrane showed increase in permeability towards
GABA.

The multiplicity of biological actions exerted
by surface - active drugs can be well explained on the
basis of liquid membrane hypothesis e.g. antihistamines
are also known[85] to have anticholinergic and local
anaesthetic action. Chlorpromazine and few other low-
potency phenothiazines have mild antihistamine[86] and
anti-serotonin[86] activity. Such actions can be explained
as a result of alteration of transport of relevant
permeants because of the liquid membrane interposed
between the permeant and the biomembrane.

The Liquid Membrane Hypothesis Vis-a-Vis Existing Theories of Drug Action :

The liquid membrane hypothesis for drug-action proposes that in a series of structurally related drugs which are congeners of a common chemical moiety and which act by reducing permeability of hydrophilic substances, any structural variation which increases hydrophobicity of the compound will increase resistance towards transport of the hydrophilic permeant. In other words, any deviation in structure leading to increase in hydrophobicity will reduce CMC of the drug, make it more potent and increase resistance towards a hydrophilic permeant. However, this sequence of events will continue so long as the hydrophilic groups of the drug responsible for interaction with the biomembrane is unaltered. Any alteration of the hydrophilic moieties of the drug may alter specificity towards the membrane and therefore may alter the nature of response towards the permeants e.g. after alteration of the hydrophilic structure, the drug may inhibit transport of another permeant more specifically than the earlier permeant. This offeres a clue towards the structure-activity relationship. An increase in hydrophobicity will alter the drug-action quantitatively i.e. it will increase the potency while change in hydrophilicity may alter the nature of action qualitatively

i.e. the specificity of the resistance towards different permeants may change. Similar comments have been made by Burger[87], in connection with structure-activity relationship.

According to rate theory[69,70,81] the dissociation rate constant can be a good indicator of the nature of action shown by a drug. An antagonist is expected to have a low dissociation constant (K_2), as compared to the agonist. Consequently, in a series of antagonists, as the dissociation constant goes on decreasing the potency of the compound as an antagonist increases. In a series of monoquaternary salts, it is indicated that $K_2 = 0.0038 \times 2.65^{12-n}$, where n = number of carbon atoms in the alkyl chain[70]. In other words, K_2 falls by a constant factor of 2.65 for each methylene group added. In case of monoquaternary salts, paton[69] has commented: "The association of these monoquaternaries is dictated by long range ionic forces, i.e., by the cationic head, but once, the molecule is bound, its dissociation is more or less hampered by Van der Waals binding of the molecule to the receptor surface. The association rate would then be similar for all the compounds, but the dissociation constant would be sensitive to length of the alkyl chain to a degree comparable with the manner in which surface tension of alkyl carboxylic acids varies with alkyl

chain length". These comments can also be understood in/terms of the liquid membrane hypothesis. The addition of each methylene group in an antagonist will increase its hydrophobicity resulting in reduction of its CMC. Lowering of CMC may be linked to increase in potency of the compound as discussed earlier. Besides reducing the CMC, an increase in the methylene groups will strengthen the hydrophobic core of the drug liquid membrane and may offer more resistance to the transport of hydrophilic permeants. The CMC of a/drug, therefore, appears to provide the same information which dissociation constant provides in case of rate theory[69].

If dose- response curve of an agonist is compared with dose-response curve of a mixture of an agonist and antagonist, it is observed that there is flattening of dose-response curve in the later case[67-70,72]. This leads to a parallel right shift in case of competitive antagonist-the proposition that agonist replaces anta- gonist is ruled out[69]. This is further substantiated by low dissociation constants in case of antagonist[69]. The observations, related to dose-response curve can also be explained on the basis of the liquid membrane hypothesis. A liquid membrane generated by a surface- active 'antagonist' drug is interposed between receptor

in the biomembrane and the agonist. As a consequence, transport of agonist is likely to be reduced resulting in lesser amount of agonist reaching the receptor. Hence to achieve the same quantum of response, higher amount of agonist will be needed. This effect will result in the shifting of dose-response curve to the right. The nature of the liquid membrane and the extent of the resistance offered to the agonist will determine the nature and extent of shift in the dose - response curve.

One experimental observation in relation to dose-response curve of agonist - antagonist mixture has necessitated the hypothesis of "spare-receptors". It is observed[70] that a mixture of an agonist and an antagonist elicits the same maximum response as in the case of agonist alone, but at a comparatively higher concentration. The dilemma is: if the receptors are occluded by the antagonist, how is it possible to obtain parallel dose-response curve with and without antagonist? or, inspite of a sizable section of   receptors being occupied by an antagonist, how can a maximal response be obtained ? The dilemma has been resolved by proposing[76] existance of "spare-receptors"; i.e., those receptors without combining with which the agonist alone was capable of eliciting maximum response. However, there is a criticism

about this hypothesis. A direct experimental demonstration of spare-receptor is still awaited[79]. Efforts have also been made to demonstrate[83] experimentally that there are no spare-receptors. Paton has commented[70] that for occupancy theory existance of spare receptors merely seems a puzzling extravagance". In the liquid membrane hypothesis for drug action, the existance of spare receptors is not necessary. The rate of transport of an agonist across the liquid membrane of an antagonist is dependent on the concentration gradient of the agonist across the liquid membrane. As concentration of the agonist is increased, the rate of flow of the agonist across the liquid membrane generated by antagonist will also increase and at a certain higher concentration of the agonist it will elicit the same quantum of response as in the absence of the antagonist. Thus, rather than existance of "hypothetical additional receptors", the resistance offered by the liquid membrane generated by the antagonist to the flow of agonist is likely to decide the strength of the biological response. An indication of this proposition is available in literature. According to the potential - svergiftung theory[89], the "action of the agonist was related to its flux across the cell membrane, which inturn was related to the driving force". The driving force is the concentration gradient.

While commenting[79] on the rate theory it is
mentioned that, in general, for the rate of action of
the drug any one of the following four steps may be
the rate determining step:

     (a) access to the receptor;

     (b) conversion of the drug from an inactive to
         active form;

     (c) rate of combination with the receptor; or

     (d) rate of production of the response;

Amongst these steps, access to the receptor
seems to be the most common rate-limiting step[79]. Hence
any event which is likely to reduce access of the agonist
to the receptor should have profound influence on nature
and sequence of agonist-receptor interaction and hence
the consequent biological response. Generation of a
liquid membrane having ability to reduce access of the
agonist to the receptor is one such step. As a result,
it is likely to affect the agonist-receptor interaction
in a notable manner.

To explain kinetics of reversible antagonism in aortic strips, a biophase model was proposed[90,91]. According to this hypothesis, it was suggested that receptors are situated in a biophase separated from the extracellular space by an interfacial barrier through which agonists (but not antagonists), penetrate quickly; penetration of this barrier is considered as the rate-limiting step dictating the kinetics of antagonism. However the existence of such a barrier in the case of antagonist has been ruled-out experimentally.[70] Another prediction of the biophase hypothesis, i.e., the dose-ratio (the ratio by which the agonist dose must be increased in order to restore a standard response in the presence of antagonist) should rise/fall exponentially when antagonist is added /removed, is also not true.[70] It is occupancy and not the dose-ratio that is observed to change exponentially. The liquid membrane hypothesis resolves this problem. Though there is no barrier for the antagonist to reach the receptor, a liquid membrane generated by an antagonist can act as a barrier to the flow of the agonist.

A general comment regarding the validity of liquid membrane hypothesis for drug action needs special mention. It is known that majority of transport processes in

biological system (especially those of neurotransmitters) are 'active' in nature. Hence only after showing that the active process is also impeded, by a drug-liquid membrane, would the role of liquid membrane phenomenon in the action of antagonistic drugs become acceptable. For any process of active transport, rate of access of the permeant to the active site is an important factor. If this itself is impeded, because of resistance to its transport, even the active transport will be reduced. This reduction can result in antagonism. This is especially true in the case of drug-receptor interaction because access to the receptor has been considered[79] to be a rate-limiting process in the whole sequence of drug action.

Thus, the liquid membrane hypothesis for drug action points out towards a new facet of drug action. This aspect of drug action has hitherto gone unnoticed. The hypothesis provides a physical basis for the action of these drugs which act by modifying the permeability of cell membranes and are surface active in nature.

The Task in this Thesis:

Since the liquid membrane hypothesis for drug action is a recently postulated one, there is a need to

carry out investigations on more categories of drugs/
biological agents, for exploring the role of liquid
membrane phenomenon in the mechanism of their biological
action with a view to further substantiating the hypothesis.
In the present thesis,therefore,an account of the studies
on   1. gonadol steriod harmones ;

2. vitamin - E (α -tocopherol);

3. prostaglandins ;

4. Anticancer drugs (5-fluorouraciland its
derivatives);

is presented.

Before an account of these studies is taken up
in ChapterIIItoVI the experimental methods used in these
studies are dercribedin a general manner in the next
Chapter (Chapter Π).

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CHAPTER II

EXPERIMENTAL - A GENERALISED VERSION

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In the present investigations three main experiments have been performed. These are ,

1. Determination of critical micelle concentrations (CMC) of the biological agents/drugs under study.

2. Determination of the liquid membrane formation in series with the supporting membrane, by the biological agents/drugs and the incorporation of these biological agents/drugs in the bio-membrane phospholipid cholesterol liquid membrane generated in series with the supporting membrane.

3. Measurements of solute permeabilities of relevant permeants through the liquid membranes generated by the drugs themselves or in association with the phospholipid and cholesterol.

Determination of the CMCs

The critical micelle concentration (CMC) of aqueous solutions of the drugs was determined from the variation of surface tension with concentration. The surface tensions were measured using a Fisher Tensiomat Model 21. The

values of the CMCs thus determined are recorded in the
relevant chapters.

Determination of Liquid Membrane Formation:

For demonstration of the formation of liquid mem-
branes by the various drugs/biological agents, in the
series with the supporting membrane, the data on hydraulic
permeability in the presence of the various concentration
of drugs/biological agents were exploited. For obtaining
the hydraulic permeability data the all glass cell dia-
grammed in Fig. 1 was utilised. The diagram of the
transport cell (Fig.1) has been well labelled to make it
self explanatory. It essentially consists of two compart-
ments C and D separated by a cellulosic (cellulose acetate/
cellulose nitrate) microfiltration membrane (Sartorious
cat. no. 11107 or 11307 pore size 0.2 μm) of thickness
1×10^{-4}m and area 2.55×10^{-5}m^2 which, infact , acts as
a supporting membrane for the liquid membranes. The stop
cock attached to compartment D could be used to adjust
the liquid miniscus in the capillary L_1L_2.

To obtain the hydraulic permeability data at various
concentrations of the drugs, aqueous solutions of the
drugs of varying concentrations were filled in the compart-
ment C of the transport cell and compartment D was filled
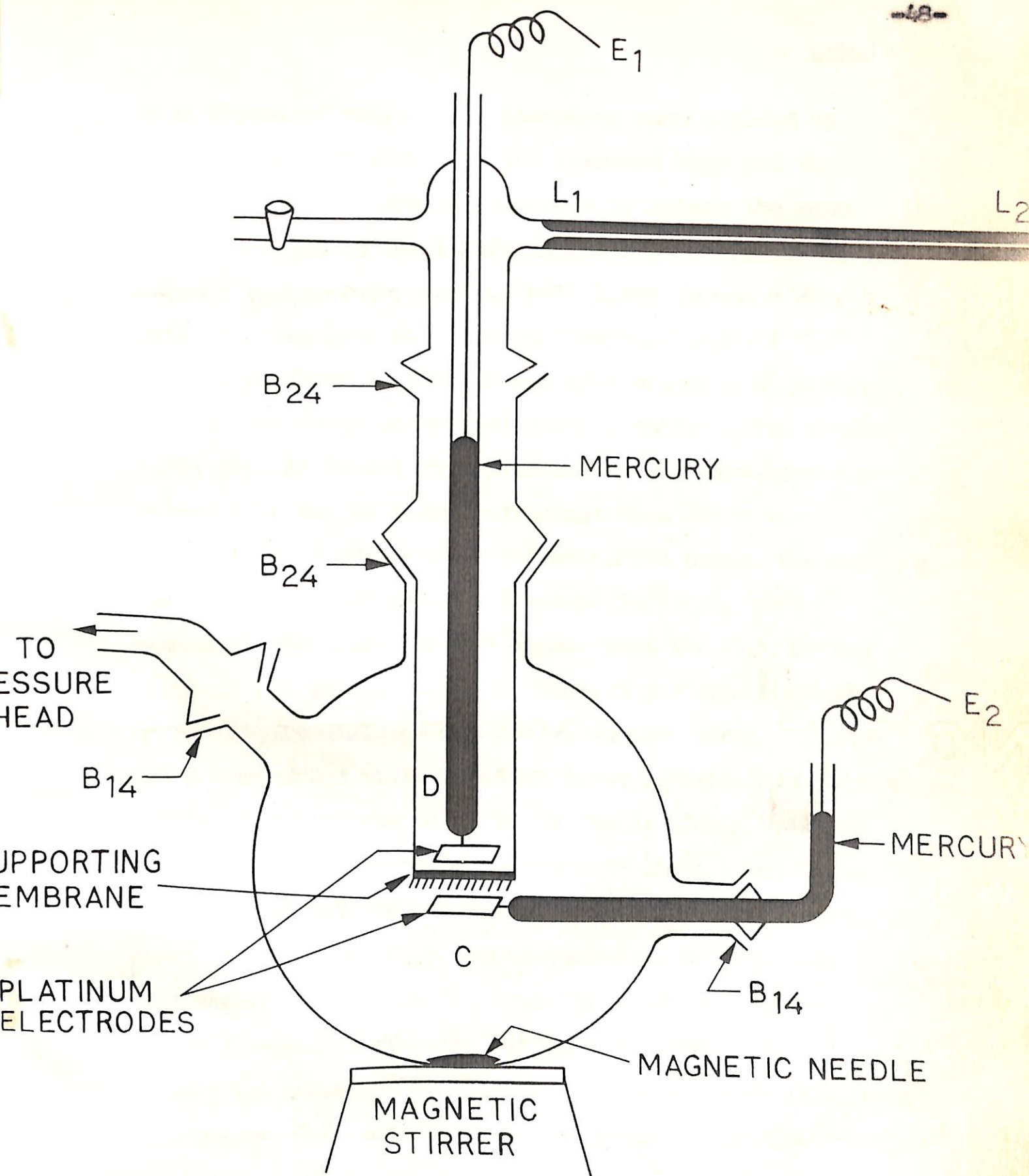
Fig.1 : The all glass transport cell

$E_1 E_2$ electrodes terminals, $L_1 L_2$ capillary.

with distilled water. known pressures were applied on
compartment C by adjusting the pressure head and the
resulting volume flux was measured by noting the rate
of advancement of the liquid miniscus in capillary L_1L_2
using a cathetometer reading upto 0.001 cm and a shop
watch reading upto 0.1 second. The magnitude of the
applied pressure difference was also measured by noting
the postion of pressure head using a cathetometer reading
0.001 cm. An important precaution in the measurement of
volume flux was to allow sufficient time after the
application of pressure on compartment C before the measure-
ment of liquid miniscus in the capillary L_1L_2 were
recorded. This was done to ensure that the flow in the
capilary was steady flow. In fact, the distance moved
by the liquid miniscus was ploted against time. If such
plots were found to be straight lines passing through the
origin, the flow was taken to be steady flow. During
volume flow measurements, solution in compartment C was
well stirred and the electrodes E_1 and E_2 (Fig.1) were
short circuited so that the electro-osmatic back flow
that could develop due to streaming potential did not
become a serious disturbing factor. The volume flux J_v
at various values of $(\triangle P)$, the applied pressure difference,
were calculated using the relation (1) where r and R are radii

$$J_v = \frac{\pi r^2 \ell}{\pi R^2 t} = \left(\frac{r}{R}\right)^2 \frac{\ell}{t} \qquad \qquad ..(1)$$

of the capillary L_1L_2 and the Membrane, M (Fig. 1) respectively and l is the distance travelled by the liquid miniscus in the capillary L_1L_2 in time t. The concentration ranges selected were such that hydraulic permeability data were obtained both below and above the CMC of the drugs.

Analysis of the Hydraulic Permeability Data:

To obtain information on the formation of liquid membrane the data on hydraulic permeability was analysed in the following manner. Volume flux J_v was plotted against applied pressure difference $\triangle P$. In all the cases the data were found to be represented by the proportional relationship

$$J_v = L_p \triangle P \qquad \qquad ...(2)$$

where L_p is the hydraulic conductivity coeffecient. The values of L_p at various concentrations of the drugs/ biological agents under study were estimated from the slopes of the J_v versus $\triangle P$ plots. The value of L_p in all cases showed a decreasing trend with the increase in the concentration of the drugs/biological agents. The decreasing trend continued upto the CMC of the drugs/ biological agents beyond which the values of L_p become more or less constant. This trend was taken to be
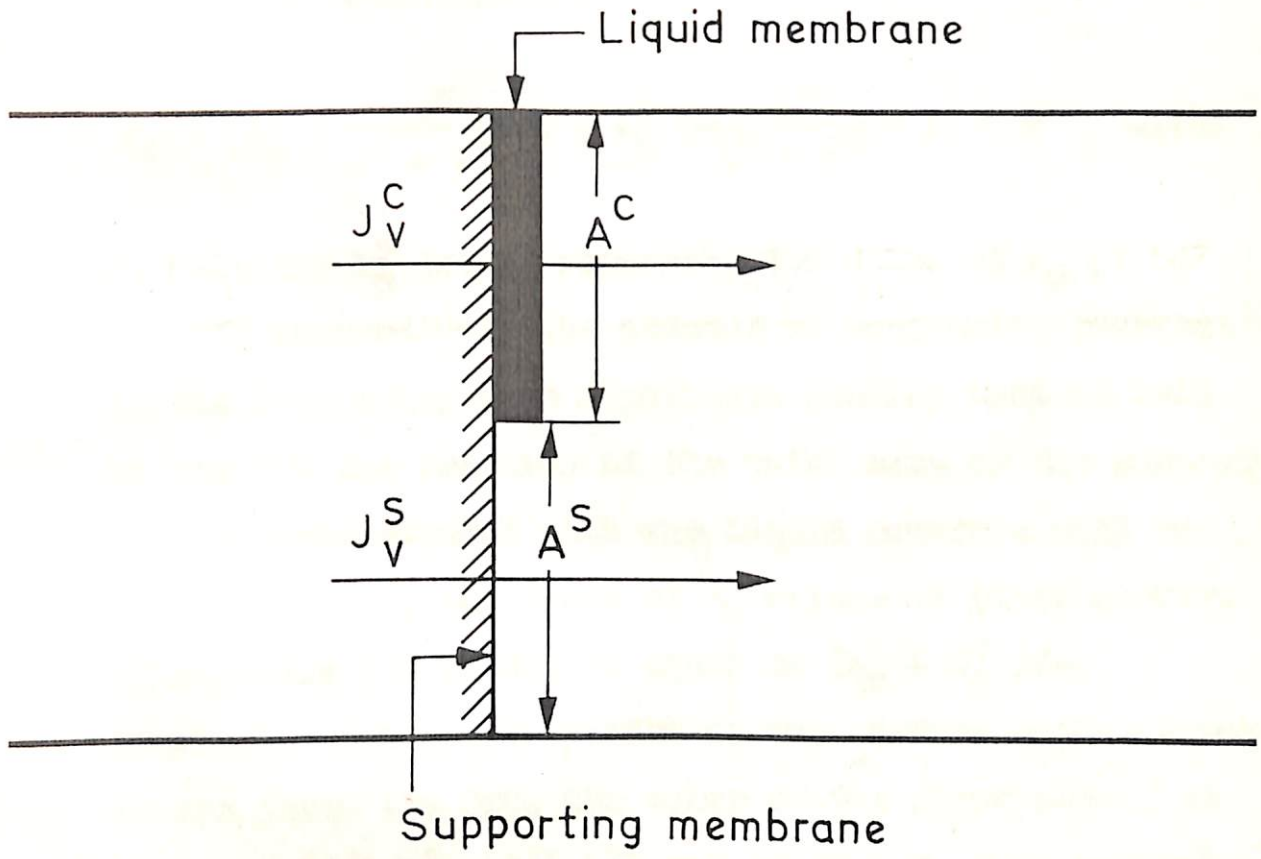
Fig.2 : The schematic representation of mosaic membrane formed when the concentration of the surfactant is lower than its CMC. J_V^S, J_V^C, A^S and A^C have the same meaning as in equation 3.

of the linear relationship between J_v and $\triangle P$, the equation (3) can be transformed into

$$J_v = \left[L_p^S \left(\frac{A^S}{A^S + A^C} \right) + L_p^C \left(\frac{A^C}{A^S + A^C} \right) \right] \triangle P \qquad ..(4)$$

Functionaly L_p^S and L_p^C represent the value of L_p at '0' and CMC respectively. The concept of progresive coverage in the liquid membrane hypothesis implies that at half of 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 J_v versus $\triangle P$ plots in view of equation (4) should be equal to $(L_p^S + L_p^C)/2$. Similarly when concentration of the surface active agent is one forth its CMC, the value of the slope should be equal to $(3/4\ L_p^S + 1/4\ L_p^C)$ and so on. Thus in general terms if the concentration of the surfactant is n times its CMC, n being less than or equal to 1, the value of the slope /J_v versus $\triangle P$/ should be equal to $\left[(1-n)\ L_p^S + n\ L_p^C \right]$. values of L_p thus computed, at various concentration of the drugs/biological agents should be in good agreement with the experimentally determined values. This agreement should constitute additional evidence in favour of liquid membrane formation.

<u>Solute Permeability Measurements:</u>

For measurement of solute permeability ω, the transport cell (Fig.1) was used. The compartment C of the transport cell was filled with the solution of desired composition of the liquid membrane generating solution (drug or drug lipid mixture) along with the solution of permeant of known concentration. The compartment D was filled either with distilled water or the liquid membrane generating solution of the same composition as was filled in the compartment C. In the control experimental no drug or biological agent under study was used.

The values of solute permeability (ω) in presence of the liquid membranes generated by the various drugs were measured using the definition[7,8] ,

$$\left(\frac{J_s}{\Delta\pi}\right)_{J_v = 0} = \omega \qquad\qquad ...(5)$$

where J_v and J_s are the volume flux and solute flux per unit area of the membrane respectively and $\Delta\pi$ is the osmotic pressure difference. The condition of no net volume flux ($J_v = 0$) during the solute permeability (ω) measurements was attained by adjusting the pressure head attached to the compartment C of the transport cell so that liquid miniscus in the capillary L_1L_2 remains

stationary. After a known period of time, which was of the order of several hours, the concentration of the permeant transported to the other compartment - compartment D was measured. The amount of the permeant gained by compartment D divided by the time and the area of the membrane, gave the value of the solute flux J_s for use in the calculation of ω using equation 5, the value of $\Delta\pi$ used in the calculation of ω was the average of the values of $\Delta\pi$ at beginning (t=0) and at the end of the experiment.

For the solute permeability measurements the composition of the liquid membrane generating solution was always above the CMC. These compositions were derived from the hydraulic permeability data.

What has been given above is the general description of the experimental procedures. The details will be recorded in the Chapters dealing with the respective studies.

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CHAPTER III

LIQUID MEMBRANE PHENOMENA IN GONADAL STEROID HORMONES

CHAPTER- III

LIQUID MEMBRANE PHENOMENA IN GONADAL
STEROID HORMONES[*]

Steroid hormones responsible for a variety of
physiological functions are reported to be surface active
in nature[1-4] and hence should be capable of interacting
with the lipid bilayer of biomembranes and modifying
permeability of biomembranes to relevant permeants. It
is quite logical to expect that this modification in the
permeabilities of relevant permeants may play role in the
physiological functions of the steroid hormones. The
model studies reported in this chapter have been conducted
with this object in view. Incidentally suggestions to
the effect that modifications in the permeability of
cell membranes brought about by steroid hormones may
play significant roles in their physiological actions,
have already been made in literature[2,5].

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* Based on this study a paper has been accepted for pub-
lication in the Indian Journal of Biochemistry and
Biophysics.

In the present study data on hydraulic permeability have been obtained to demonstrate, using Kesting's hypothesis[6], formation of liquid membrane by sphingomyelin in series with a supporting membrane and incorporation of steroid hormones in them. Since entire hypothalamus is under the influence of neurotransmitters in the release of hypophysial hormones[7-9] including gonadal steroid hormones responsible for a variety of physiological functions, transport of neurotransmitters viz, adrenaline, noradrenaline, dopamine and serotonin through the liquid membranes generated by the steroid hormones in association with sphingomyelin has been studied. The data obtained on the modification in the permeabilities of neurotransmitters in the presence of the liquid membranes have been discussed in the light of the various physiological actions of the steroid hormones.

The present studies were conducted on ethinylestradiol, progesterone and testosterone propionate.

Materials and Methods

Materials:

Sphingomyelin and progesterone (Patel Chest Institute, CSIR Centre for Biochemicals, Delhi), ethinylestradiol and testosterone propionate (Roussel Pharmaceuticals

(India) Bombay), L-adrenaline hydrogen tartarate and dopamine chlorhydrate (both from Loba Chemie), L-noradrenaline (Fluka-AG), 5-hydroxy tryptamine creatinine sulphate (Serotonin) (Koch-Light Laboratories) and triple distilled water distilled in all pyrex glass still were used in the present experiments.

Aqueous solutions of desired concentration of sphingomyelin and of mixtures of sphingomyelin and steroid hormones of desired composition were prepared by adding necessary volume of ethanolic stock solutions of known concentrations to aqueous phase with constant stirring. In the aqueous solutions thus prepared the final concentration of ethanol was never allowed to exceed 0.1% by volume because it was experimentally shown that the surface tension of a 0.1% aqueous ethanol was more or less equal to that of water. The aqueous solutions of adrenaline, noradrenaline, dopamine and serotonin (5HT) were prepared the usual way.

The critical micelle concentrations (CMCs) of aqueous solutions of Sphingomyelin, ethinylestradiol, progesterone and testosterone propionate as determined from the variation of their surface tensions with concentrations were found to be 16 ppm, 2.70×10^{-7}M,

9.0×10^{-5}M and 3.87×10^{-6}M respectively. Surface tenstions were measured using a Fisher tensiomat model 21.

The all glass cell described earlier (Chapter II) was used for transport studies. A Sartorius cellulose acetate microfiltration membrane (Cat. no. 11107, pore size 0.2 µm) of area 2.55×10^{-5}m^2 and thickness 1×10^{-4}m, which acted as supporting membrane for the liquid membranes separated the transport cell into two compartments C and D (Fig. 1   Chapter II).

To obtain the hydraulic permeability data, solutions of varying composition of sphingomyelin - steroid hormone mixture were filled in compartment C of the transport cell (Fig. 1 ,Chapter II) and compartment D was filled with water. The method used for the hydraulic permeability measurements was the same as described in Chapter II.

For solute permeability (ω) measurements for various permeants namely, adrenaline, noradrenaline, dopamine and serotonin solutions of known concentrations of the permeant prepared in the aqueous solutions of the mixtures of sphingomyelin and one of the steroid hormones were filled in compartment C of the transport cell and

compartment D was filled with distilled water. The composition of the aqueous solutions of the sphingomyelin-steroid hormone mixtures used in the solute permeability (ω) experiments were those at which the liquid membrane generated by sphingomyelin completely covers the supporting membrane and is saturated with the steroid hormone. In fact these compositions were derived from the hydraulic permeability data. The methods used for solute permeability measurements have already been described in Chapter II.

All measurements were made at constant temperature using a thermostat set at $37 \pm 0.1°C$.

Estimations:

The amounts of the various permeants viz., adrenaline, noradrenaline, dopamine and serotonin, transported to compartment D were estimated spectrophotometrically by measuring absorbance at 282.4 nm[10]. A varian Cary 17-D spectrophotometer was used for absorbance measurements.

Results and Discussion:

The hydraulic permeability data at various concentrations of sphingomyelin were found to be obeyed by the proportional relationship

$$J_v = L_p \triangle P \qquad \qquad \ldots(2)$$

where J_v is the volume flux per unit area of the membrane,
$\triangle P$ is the applied pressure difference and L_p is the
hydraulic conductivity coefficient. The values of L_p at
various concentrations of sphingomyelin, estimated from
the J_v versus $\triangle P$ plots showed a decreasing trend with
the increase in concentrations of sphingomyelin upto
CMC beyond which they became more or less constant
(Table 4). This trend is in keeping with the Kesting's
liquid membrane hypothesis[6] according to which as mentioned
in Chapter II when a surfactant is added to aqueous
phase, the surfactant layer which forms spontaneously at
the interface acts as a liquid membrane and modifies
mass transfer across the interface. As concentration
of the surfactant is increased the interface gets pro-
gressively covered with the surfactant layer liquid
membrane and at the CMC it is completely covered. Analysis
of the transport data in light of mosaic model[11-13]
further substantiates the formation of the liquid membrane
in series with the supporting membrane. As shown in
Chapter II when concentration of the surfactant is n
times its CMC, $n \leq 1$, the value of L_p should be
$\left[(1-n)L_p^s + n\, L_p^c\right]$ where the superscripts s and c
respectively represent the values of L_p for 0 and the CMC

of the surfactant. The values of L_p thus calculated Compare favourably with the experimentally determined values (Table 1).

The hydraulic permeability data at varying concentrations of steroid hormones in the sphingomyelin steroid mixtures in which the concentration of sphingomyelin was kept constant at 16 ppm (CMC), were utilised to gather information about the incorporation of the steroid hormones in the liquid membrane generated by sphingomyelin in series with the supporting membranes. These data were also found to be represented by the proportional relationship (2). The concentration 16 ppm of sphingomyelin used in these experiments is the concentration at which the liquid membrane generated by sphingomyelin completely covers the supporting membrane (Table 1). The values of L_p, at various concentrations of steriod hormones, in the sphingomyelin-steroid mixtures, estimated from the J_v versus ΔP plots showed a decreasing trend with the increase in concentration of the steroid hormones upto a certain concentration beyond which they became more or less constant (Table 2). The decreasing trend in the values of L_p is due to incorporation of steroid hormones in the sphingomyelin liquid membrane. These concentrations beyond which the values of L_p become more or less constant are the concentrations at which the sphingomyelin liquid membranes present at the

TABLE - 1

Values of L_p^a at Various Concentration of Sphingomyelin

Concentration (ppm)	0.00	4 ppm	8 ppm	12 ppm	16 ppm	32 ppm
$L_p^b \times 10^8 (m^3 s^{-1} N^{-1})$	2.754 ±0.061	2.417 ±0.052	2.016 ±0.030	1.616 ±0.020	1.247 ±0.010	1.239 ±0.019
$L_p^c \times 10^8 (m^3 s^{-1} N^{-1})$	-	2.377 ±0.048	2.001 ±0.035	1.637 ±0.036		

[a] The values are reported as arithmetic mean of 10 repeats ± S.D.

[b] Experimental Values

[c] Computed values using mosaic model.

interface are completely saturated with the steroid hormones.

The compositions of the sphingomyelin-steroid hormone mixtures used in the solute permeability (ω) experiments were derived from this study (Table 2). It is logical to expect that hydrophobic ends of the molecules in the liquid membranes would be preferentially oriented towards the hydrophobic supporting membrane and the hydrophilic ends would be drawn outwards away from it.

Solute Permeability Data and Physiological Actions:

The values of solute permeability (ω) of the various biogenic amines in the presence of liquid membranes generated by sphingomyelin-steroid hormone mixtures are recorded in Table 3. The trends in the modification in the values of solute permeability (ω) observed in the present model studies can be seen to be in agreement with those reported on biological cells/tissues (Table 4). The modifications in the values of solute permeabilities (ω) of the various neurotransmitters (Table 3) appear relevant to various physiological functions of the steroid hormones.

It is well known that in response to a hypophysiotropin, adenohypophysial hormones and the gonadotropins

TABLE 2

Values of L_p^a at Various Concentrations of Gonadal Steroids in Sphingomyelin Gonadal Steroid Mixtures[b]

	Ethinyl Estradiol				
Concentration x 10^6M	0.00	1.00	1.50	2.00	3.00
L_p x 10^8 ($m^3s^{-1}N^{-1}$)	1.247 ±0.010	0.8311 ±0.036	0.6012 ±0.022	0.5975 ±0.018	0.5938 ±0.010

	Progesterone				
Concentration x 10^6M	0.00	0.20	0.30	0.40	0.50
L_p x 10^8 ($m^3s^{-1}N^{-1}$)	1.247 ±0.010	0.9680 ±0.010	0.8394 ±0.0024	0.7000 ±0.002	0.6972 ±0.001

	Testosterone Propionate			
Concentration x 10^6M	0.00	1.00	1.50	2.00
L_p x 10^8 ($m^3s^{-1}N^{-1}$)	1.247 ±0.01	0.734 ±0.005	0.4877 ±0.061	0.4533 ±0.012

[a] The values are reported as arithmetic mean of 10 repeats ± S.D.

[b] Concentration of sphingomyelin kept constant at 16 ppm

TABLE -3

Solute Permeability (ω)[a] of Various Permeants in Presence of Sphingomyelin Gonadal Steroid Mixtures

	$\omega_0 \times 10^{10}$ (mol s^{-1}N^{-1})	$\omega_1 \times 10^{10}$ (mol s^{-1}N^{-1})	$\omega_2 \times 10^{10}$ (mol s^{-1}N^{-1})	$\omega_3 \times 10^{10}$ (mol s^{-1}N^{-1})
Adrenaline[b]	2.050±0.043	0.855±0.053	1.151±0.022	1.481±0.01
Noradrenaline[b]	4.467±0.192	2.775±0.062	4.275±0.056	3.160±0.087
Dopamine[b]	3.266±0.097	2.825±0.014	4.394±0.069	2.892±0.088
Serotonin[b]	3.189±0.089	2.579±0.181	3.771±0.128	3.612±0.159

[a] Values of ω are reported as arithmetic mean of 10 repeats ± S.D.

[b] Initial concentrations: adrenaline = 3.000 x 10^{-5} mol/lit. noradrenaline = 5.889 x 10^{-5} mol/lit, dopamine = 5.273 x 10^{-5} mol/lit, Serotonin = 2.466 x 10^{-5} mol/lit.

ω_0 Control value when sphingomyelin alone was used (spingomyelin concentration = 18 ppm).

ω_1 Values in the Presence of spingomyelin-Ethinyl estradiol mixture of composition 18 ppm with respect to spingomyelin and 2.0x10^{-6}M with respect to ethinyl estradiol.

ω_2 values in the presence of sphingomyelin-progesterone mixtures of composition 18 ppm with respect to spingomyelin and 2.0 x 10^{-6}M with respect to progesterone.

ω_3 Values in the presence of sphingomyelin-testosterone propionate mixtures of composition 18 ppm with respect to sphingomyelin & 2.0 x 10^{-6}M with respect to Testosterone Propionate.

TABLE - 4

Comparison of the Trends in Values of Solute Permeability (ω)
of Neurotransmitters in the Presence of Sphingomyelin-Steroid
Liquid Membranes with those Reported on Biological Cells/Tissues

Trends reported on biological cells/tissues	Trends observed (this work)
1. Application of estradiol and progesterone to hypothalmic slices inhibits uptake of noradrenaline[37]	Permeability of noradrenaline is impeded in presence of both estrogen and progesterone.
2. Gonadal secretions decrease cerebral noradrenaline turnover which is increased following castration[38-42]	Permeability of noradrenaline is impeded in the presence of testosterone.
3. Progesterone treatment increases turnover in basal forebrain nuclei[38]	Permeability of dopamine increases in the presence of progesterone.
4. Re uptake of serotonin in midbrain and pons/medulla following estrogen treatment[43]	Permeability of serotonin decreases in the presence of estrogen.

5. Estrogen applied to hypo-
   thalmic slices inhibits
   the uptake of serotonin[37]

Permeability of serotonin
decreased in the presence of
estrogen.

6. Progesterone treatment
   increases serotonin levels
   in mid brain and hind brain[44],
   Progesterone treatment increa-
   ses serotonin turnover in
   septum, raphae. and hypotha-
   lmus[45].

Permeability of serotonin
is enhanced in the presence
of progesterone.

7. Longer treatment with estro-
   gen depressed the adrenaline
   content in rat uterine
   extracts[46].

Permeability of a_____
adrenaline decreases in
the presence of estrogen.

8. Estrogen treatment caused
   depletion of noradrenaline
   concentration in uterine
   periarterial nerves and in
   uterine extracts[47].

Permeability of noradrenaline
is lowered in the presence
of estrogen.

9. Estrogen concentrations at micromolar range block catecholamine uptake in isolated rat heart[48].	Permeability of catecholamine is reduced in the presence of estrogen.
10. Estrogen application to prolactin secreting pituitary cells in culture blocks dopamine action[49].	Permeability of dopamine is reduced in the presence of estrogen.

are released which stimulate their target tissues. Target
tissue stimulation results in increased secretion of
target tissue hormones such as thyroid hormones, adrenal
glucocorticoids and gonadal steroid hormones. These
hormones then in addition to acting on their respective
target tissues to mediate their _____ actions also act
on higher brain centres, hypothalmus and pituitary and
exercise a negative feed back control.

Recently biogenic amines particularly dopamine,
noradrenaline and serotonin have been implicated in
the feed back mechanism[7,14-16]. For example dopamine
has been shown to cause the release of LH/FSH-RH and P-RIH.
The release of LH/FSH-RH produced by intraventricularly
injected dopamine is blocked by the previous intraven-
tricular injection of estradiol[7]. The impediment in the
transport of dopamine in the presence of the liquid
membranes generated by the sphingomyelin-ethinyl estradiol
mixture as observed in the present study (Table 3)
appears to be a contributing factor to the negative feed

LH/FSH-RH - Luteinizing hormone/Follicle stimulating
            hormone-releasing hormone

P-RIH   - Prolactin - release inhibiting hormone.

back mechanism. It is also documented[7] that patients treated with drugs like reserpine and chlorpromazine which have been shown to impede the transport of biogenic amines including dopamine[10,17] display evidence of altered pituitary functions, e.g., failure to ovulate. Since there is evidence of dopamine being directly released into portel vessels and acting on pituitary.[14] this observation on the altered pituitary functions in the patients treated with the drugs like reserpine and chlorpromazine is consistent with the conclusion that reduction in the permeability of dopamine due to the ethinyl estradiol liquid membrane formed in association with sphingomyelin is a contributing factor in the negative feed back mechanism. It is also documented that implantation of testosterone in the median eminence of rats inhibits pituitary gonadotropin secretion[18] by decreasing the level of gonadotropin-releasing hormones[8]. The reduced permeability of biogenic amines like dopamine in the presence of sphingomyelin - testosterone mixture (Table 3) could be a plausible explanation for this observation.

At hypothalmic level, the secretion of prolactin in mammals is controlled by the inhibitory hormone P-RIH and possibly by a prolactin releasing hormone, P-RH,

the role if any of P-RH is only of secondary importance[7]
The release of P-RIH from neuroendocrine transducer
cells in the median eminence is controlled by hypo-
thalamic dopamine and it has been shown that drugs
like reserpine, chlorpromazine and haloperidol which
reduce the permeability of dopamine[10,17,19] and there-
by lower its concentration in hypothalamic region
decrease the release of P-RIH and thereby promote
prolactin release[20]. Since ethinyl estradiol was found
to reduce the permeability of dopamine (Table 3) it
should have effects similar to that of reserpine,
chlorpromazine and haloperidol which indeed is the case[20].
It is reported that disorders like galactorrhoea or
gynaecomastia may also arise from estrogen secreting
tumors and also as a side effect of oral contraceptives[7].

MSH_s secretion by the pars intermedia of the
pituitary gland are reported to be under the control
of catecholamines viz adrenaline, noradrenaline and
dopamine[21]. Drugs such as, reserpine, haloperidol and

P-RH   - Prolactin releasing hormone

MSH   - Melanocyte - stimulating hormones

chlorpromazine which block the actions of catechol amines[10,17 & 19] are reported to stimulate MSH secretion.[21] Karkun and Sen (1965)[22] reported increased pituitary levels of MSH in ovariectomized rats after estrogen treatment for thirty days. These observations are consistent with the reduced permeability of catecholamines in the presence of ethinyl estradiol as observed in the present study (Table 3).

Dopamine, noradrenaline and serotonin have been reported to increase growth hormone release in animals and man[15]. Dopaminergic drugs increased growth hormone release whereas inhibitors of dopamine activity such as haloperidol, reserpine etc., caused reduction in the release of growth hormones. Noradrenaline and its agonists are also reported to promote growth hormone release[15]. The role of serotonin on the release of growth hormone is however controversial. It is documented that estrogens have been used to limit stature in girls with excessive predicted height and to decrease growth activity in acromegalics[23]. It has also been documented that excess androgen secretion early in adolescent development can lead to shortened stature[24]. Reduction in the permeability of biogenic amines particularly dopamine and noradrenaline in the presence of liquid membranes generated by

both, ethinyl estradiol and testosterone in association with sphingomyelin (Table 3) leading to reduced secretion of growth hormones could also be a plausible explanation for these observations.

Neurohypophysial secretions containing ADH, oxytocin and neurophysins are evoked by different stimuli. The nerve cell bodies of the paraventricular and supraoptic nuclei have both cholinergic and noradrenergic nerve endings impringing on them. Thus the activity in the neurosecretory cells is perhaps controlled by acetylcholine and noradrenaline. Acetylcholine and nicotine injected into carotid circulation cause the release of ADH, oxytocin and neurophysins while noradrenaline inhibits their release[7,25]. Ovarian hormones are reported to facilitate the release of neurohypophysial hormones during appropriate physiological stimuli e.g. mating. The present data on the reduced permeability of noradrenaline in the presence of the mixtures of the gonadal steroid hormones and sphingomyelin (Table 3) appear to indicate that access of noradrenaline to the post synaptic receptor may be

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ADH   - Antidiuretic hormone.

reduced due to the resistance offered by the liquid membranes of these steroids in association with membrane phosphalipids. This reduction in the access of noradrenaline is likely to reduce its inhibitory effect and thereby facilitate the release of the neurohypophysial hormones.

Estrogens are reported to have strong antidopaminergic action[3]. For example estrogen possesses neuroleptic like quality and potentiates neuroleptic induced parkinsonism[3,26]. Since reduced concentration of dopamine and serotonin in brain have been linked with neuroleptic actions and extrapyramidal symptom like parkinsonism arising from it[19,27 & 28], it appears that impediment in the transport of both dopamine and serotonin in the presence of estrogen/sphingomyelin liquid membrane as observed in the present study (Table 3) may be one of the contributing factors to these effects.

Antidepressant drugs like imipramine are known to act by reducing the uptake of biogenic amines,[29] particularly noradrenaline and serotonin[20]. The reported antidepressant effects of estrogen[5,30] may also be assigned to the reduced permeability of biogenic amines particularly noradrineline and serotonin (Table 3)

in the presence of sphingomyelin - estrogen liquid membranes. Progesterone on the contrary is reported[31] to have a depressant effect and also an anaesthetic effect. In fact the anaesthetic effect of progesterone has been interpreted as an exaggerated expression of its depressant effect[31]. The depressant action of progesterone could also be assigned to enhanced permeability of serotonin in the presence of spingo-myelin-progesterone liquid membrane (Table 3).

Reduction in concentration of serotonin at the postsynaptic receptor resulting in defective neurotransmission has been implicated in migrain[32]. Premenstrual migrainous headache is reported to be aggravated by oral contraceptives and initiated by switching to a progestogen only preparation[31,33,34]. The rationale for these observations may also lie in reduction and enhancement respectively in the permeability of serotonin in the presence of liquid membranes generated by estrogen and progesterone in association with sphingomyelin (Table 3).

Noradrenaline and serotonin are reported to have profound effect on body temperature and evoke thermoregulatory responses when they are injected into the anterior hypothalamus or the cerebral ventricles of

experimental animals. It has been shown that the intracerebroventricular injection of serotonin in cat, dog and monkey produces rise in body temperature whereas noradrenaline produces a fall in body temperature. A convenient index of ovulation is the change in body temperature which occurs after ovulation and which is due to increased progesterone levels in the blood[31, 35&36]. Enhancement in the permeability of serotonin and reduction in the permeability of noradrenaline in the presence of sphingomyelin-progesterone liquid membranes as observed in the present study (Table 3) could also be a contributing factor to the thermogenic effects of progesterone.

Thus it appears that modification in the permeability of neurotransmitter molecules in the presence of gonadal steroid hormones-brain phospholipid liquid membranes may play a significant role in the physiological functions of the steroid hormones.

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CHAPTER IV

LIQUID MEMBRANE PHENOMENA IN PROSTAGLANDINS -
STUDIES ON PROSTAGLANDIN E$_1$ AND PROSTAGLANDIN F$_{2\alpha}$

CHAPTER - IV

LIQUID MEMBRANE PHENOMENA IN PROSTAGLANDINS -
STUDIES ON PROSTAGLANDIN E_1 AND PROSTAGLAN-
DIN $F_{2\alpha}$.*

The prostaglandins are among the most prevalent
autocoids and have been detected in almost every tissue
and body fluid; they produce in minute amounts, a
remarkably broad spectrum of effects that embrace
practically every biological function. No other auto
coids show more numerous and diverse effects than do
prostaglandins.

Just by looking at the structures of prostaglandins
their surface - active nature becomes apparent prima facie.
One can, therefore suspect that prostaglandins, when added
to an aqueous phase, according to Kesting's liquid
membrane hypothesis[1] would generate surfactant layer
liquid membranes at the interface.

In the present studies the data on hydraulic
permeability in the presence of various concentrations
of the prostaglandins have been obtained to demonstrate

the formation of the surfactant layer liquid membrane, they generate in series with a supporting membrane. The data on the hydraulic permeability in the presence of varying concentrations of the prostaglandins in a mixture of lecithin and cholesterol of fixed composition have been utilized to demonstrate incorporation of the prostaglandins into the liquid membrane generated by the lecithin - cholesterol mixture. Transport of several relevant permeants through the liquid membranes, generated by the lecithin - cholesterol - prostaglandin mixtures in series with a supporting membrane has been studied and the data obtained have been discussed in light of the reported biological effects of the prostaglandins. In these model studies a Sartorius cellulose nitrate microfiltration membrane was deliberately Chosen as supporting membrane to highlight the role of passive transport through the liquid membrane in the pharmacological actions of prostaglandins.

Materials and Methods:

Materials:

Lecithin (Phosphatidylcholine from egg yolk) and progesterone (Patel Chest Institute, CSIR Centre for Biochemicals, Delhi), Cholesterol (Centron Research

were in agreement with the values determined earlier[2,3].
The surface tensions were measured using a Fisher
Tensiomat Model 21.

Methods :

The all glass cell described in Chapter II was
used for the transport studies. It essentially consisted
of two compartments C and D separated by a sartorius
cellulose nitrate microfiltration membrane (catalog No.
11307) 1 x 10^{-4}m thick and 2.55 x 10^{-5} m^2 in area which
acted as supporting membrane for the liquid membranes.

To obtain the hydraulic permeability data which
were utilized to demonstrate the formation of liquid
membranes by prostaglandins, compartment C of the
transport cell, Fig. 1 of Chapter II, was filled with
aqueous solutions of varying concentrations of prostag-
landins, PGE$_1$ or PGF$_{2\alpha}$ and compartment D was filled with
distilled water, known pressures were applied to compart-
ment C and the consequent movement of the liquid
miniscus in the capillary L$_1$L$_2$ of known diameter (Fig. 1
of Chapter II) was measured using a cathetometer reading
upto 0.001 cm and a stop-watch reading upto 0.1s.,
During the hydraulic permeability measurments the electro-
des E$_1$ and E$_2$ (Fig. 1 of Chapter II) were short circuited

so that the electroosmotic backflow due to the streaming potential developed across the membrane did not interfere with hydraulic permeability measurments.

Similarly, to obtain the hydraulic permeability data which were utilized to demonstrate the incorporation of prostaglandins into the liquid membranes generated by lecithin-cholesterol mixtures, compartment C of the transport cell was filled with solutions of varying concentrations of prostaglandins prepared in the aqueous solutions of the mixture of lecithin and cholesterol of fixed composition (15.542 ppm with respect to lecithin and 1.175×10^{-6}M with respect to cholesterol) and compartment B (Fig. 1 of Chapter II) was filled with distilled water. This particular composition i.e., 15.542 ppm with respect to lecithin and 1.175×10^{-6}M with respect to cholesterol, of the lecithin-cholesterol mixture was chosen for preparing the solutions of varying concentrations of prostaglandins because it has been shown in earlier studies [2-4] that at this composition the liquid membrane generated by lecithin completely covers the interface and is fully saturated with cholesterol.

For solute permeability (ω) measurement for relevant permeants the method out-lined earlier (in chapter II) was used. Compartment C of the transport cell (Fig.1 of Chapter II) was filled with the solution of known concentration of the permeants prepared in the aqueous solution of lecithin, cholesterol and one of the prostaglandins (PGE_1 and $PGF_{2\alpha}$) under study and compartment D was filled with distilled water. The composition of the aqueous solution of the lecithin-cholesterol-prostaglandin mixture used in the solute permeability experiments was such that the liquid membrane generated by lecithin, in series with the supporting membrane, was completely saturated with both cholesterol and the prostaglandin under study. This composition was derived from one of our earlier study[3] and from present data on hydraulic permeability in the presence of varying concentrations of prostaglandins in the lecithin - cholesterol mixture of fixed composition. Since lecithin, cholesterol, and prostaglandins are all surface active in nature, i.e., they have both hydrophilic and hydrophobic parts in their structure, it is obvious that in the liquid membranes generated in the solute permeability experiments, the hydrophobic tail of these molecules will be oriented preferentially toward the hydrophobic supporting membrane and the hydrophilic moieties will be drawn away from it.

All measurements were made at constant temperature using a thermostat set at $37 \pm 0.1°C$.

The amounts of the various permeants transported to compartment D were estimated as follows:

(i) <u>Glucose</u>. The amount of glucose was estimated using dinitrosalicylic acid. The reaction is followed by measuring the extinction at 540 nm[5].

(ii) <u>Histamine</u>. The amount of histamine was estimated by a method involving condensation of histamine with O-phthaldehyde to yield a highly fluorescent product[6].

(iii) <u>Adrenaline</u>. The amount of adrenaline (hydrogen tartarate) was estimated by UV absorption at 291 nm in 0.1N hydrochloric acid[7].

(iv) <u>Ethinyl estradiol</u>. The amount of ethinyl estradiol was estimated by a chemical method[8] using quinol - H_2SO_4 as a coloring reagent and the color developed was measured at 538 nm using a spectrophotometer.

(v) <u>Progesterone</u>. The amount of progesterone was estimated using a Cary 17-D spectrophotometer

at 242 nm, the absorption maxima for progesterone[9].

(vi) <u>Amino acids</u>. The amounts of glycine and γ-amino butyric acid (GABA) were estimated from the amount of their reaction products with ninhydrin measured at 570 nm[10].

(vii) <u>Cations</u>. The amounts of sodium and potassium ions were determined using a flamephotometer (Model CL-22, Elico).

RESULTS AND DISCUSSION

The hydraulic permeability data at various concentrations of prostaglandins- both PGE_1 and $PGF_{2\alpha}$ in all the cases presently studied were found to be represented by the proportional relationship,

$$ J_v = L_p \triangle P \quad . $$

where J_v represents the volume flux per unit area of the membrane, $\triangle P$ is the applied pressure difference, and L_p stands for the hydraulic conductivity co-efficient.

The values of L_p at various concentrations of the two prostaglandins estimated from the slopes of the J_v versus $\triangle P$ plots are recorded in tables I and II.

TABLE I

Values of L_p at Various Concentrations of Prostaglandin E_1 and Prostaglandin $F_{2\alpha}$

	Concentration ($\times 10^8$ M)						
		0.2	0.4	0.6	0.8	1.0	
	0.0	(0.2 CMC)	(0.4 CMC)	(0.6CMC)	(0.8CMC)	(1CMC)	1.6	2.0
Prostaglandin E_1								
$L_p^a \times 10^8 (m^3 s^{-1} N^{-1})$	5.971 ±0.085	5.616 ±0.065	5.082 ±0.122	4.746 ±0.054	4.355 ±0.050	3.851 ±0.049	3.794 ±0.028	3.771 ±0.095
$L_p^b \times 10^8 (m^3 s^{-1} N^{-1})$		5.547 ±0.078	5.123 ±0.070	4.699 ±0.063	4.275 ±0.056	-	-	-

		2.32	4.65	6.97	9.30		
	0.0	(0.25CMC)	(0.5CMC)	(0.75CMC)	(1CMC)	18.60	27.90
Prostaglandin $F_{2\alpha}$							
$L_p^a \times 10^8 (m^3 s^{-1} N^{-1})$	5.971 ±0.085	4.958 ±0.141	4.016 ±0.125	3.067 ±0.086	1.997 ±0.137	1.930 ±0.051	1.907 ±0.105
$L_p^b \times 10^8 (m^3 s^{-1} N^{-1})$	-	4.977 ±0.131	3.984 ±0.134	3.013 ±0.115	-	-	-

[a] Experimental values.

[b] Calculated values using mosaic model

TABLE II

Values of L_p at Various Concentrations of Prostaglandin E_1 and Prostaglandin $F_{2\alpha}$ in Lecithin-Cholesterol Mixtures[a]

	Concentration ($\times 10^8$M)					
	0.0	0.2	0.4	0.6	0.8	1.0
Prostaglandin E_1						
$L_p \times 10^8 (m^3 s^{-1} N^{-1})$	5.550 ± 0.136	5.158 ± 0.169	4.824 ± 0.116	4.529 ± 0.106	4.640 ± 0.092	4.600 ± 0.055
	0.0	2.32	4.65	6.97	9.30	18.60
Prostaglandin $F_{2\alpha}$						
$L_p \times 10^8 (m^3 s^{-1} N^{-1})$	5.550 ± 0.136	4.995 ± 0.028	4.555 ± 0.035	4.087 ± 0.025	4.055 ± 0.025	4.055 ± 0.058

[a] Lecithin and cholesterol concentrations kept constant at 15.542 ppm and 1.175×10^{-6}M, respectively.

The values of L_p progressively decrease with the increase in the concentration of the prostaglandin upto their CMCs, beyond which they become more or less constant. This trend in the values of L_p is in accordance with Kesting's liquid membrane hypothesis and is indicative of the formation of liquid membranes by the prostaglandins in series with the supporting membrane. The values of L_p were further analysed in the light of the mosaic model[11-13] to obtain additional evidence in favour of the formation of liquid membrane in series with the supporting membrane. It has been shown in Chapter II that if the concentration of the surfactant is n times its CMC, $n \leqslant 1$, the value of L_p should be equal to

$$[(1-n) L_p^s + n L_p^c] \quad .$$

where the superscripts s and c represent the values for the bare supporting membrane and the supporting membrane completely covered with the surfactant layer liquid membrane, respectively. Functionally L_p^s and L_p^c represent the values when the surfactant concentration equals to/0 and CMC respectively. The values of L_p thus computed at several concentrations of two prostaglandins below their CMCs match with/the corresponding experimental values (Table I) lending further evidence,

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infavour of the formation of liquid membrane by the prostaglandin in series with the supporting membrane.

Information on the in corporation of prostaglandins into the liquid membrane generated by the lecithin-cholesterol mixture can be obtained from the hydraulic permeability data (Table II) at varying concentrations of the prostaglandins in the lecithin cholesterol mixture of fixed composition - 15.542 ppm with respect to Lecithin and 1.175×10^{-6}M with respect to cholesterol. The data (Table II) reveal that as the concentration of the prostaglandin is increased, holding the concentration of lecithin and cholesterol constant, the value of L_p, which measures the reciprocal of the resistance to volume flow decreases. The decreasing trend in the values of L_p continues upto a PGE_1 concentration equal to 0.6×10^{-8}M and a $PGF_{2\alpha}$ concentration equal to 6.97×10^{-8}M, and thereafter the values of L_p become more or less constant. This trend in the values of L_p (Table II) is indicative of the strengthening of the hydrophobic core of the liquid membrane generated by the lecithin-cholesterol mixture at the interface due to its incorporation with the prostaglandins. It is also apparent from the data (Table II) that at a concentration equal to

0.6×10^{-8} M the lecithin - cholesterol liquid membrane is saturated with PGE_1 and at concentration equal to 6.97×10^{-8} M the lecithin -cholesterol liquid membrane is saturated with $PGF_{2\alpha}$. Inorder to ascertain whether the added prostaglandin reaches straight to the interface or not, surface tensions of solutions of various concentrations of the prostaglandins - both PGE_1 and $PGF_{2\alpha}$ - prepared in the aqueous solutions of lecithin - cholesterol mixtures of composition 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 the lecithin - cholesterol mixture showed a further decrease upon addition of the prostaglandins. The decreasing trend in the values of the surface tension continue to a 0.6×10^{-8} M concentration in the case of PGE_1 and to a 6.97×10^{-8} M concentration in the case of $PGF_{2\alpha}$. This trend indicates that the added prostaglandins, both PGE_1 and $PGF_{2\alpha}$, reach deep into the interface of the liquid membranes generated by the lecithin-cholesterol mixture in series with the supporting membrane.

Solute Permeability Data

The data, recorded in Table III on the solute permeability (ω) of various permeants in the presence of the liquid membrane generated by [PGE₁ and]PGF$_{2\alpha}$ in association with the lecithin - cholesterol mixture, appear relevant to the various reported pharmacological actions of the prostaglandins. The values in Table III are expressed as arithmatic mean ± standard deviation based on 15 repeats for each value of ω . The present data (Table III) show that the solute permeability (ω) for glucose is increased in the presence of both PGE₁ and PGF$_{2\alpha}$, the increase in the presence of PGE₁ being much larger than the increase in the presence of PGF$_{2\alpha}$. This observation on the increase in permeability of glucose is consistant with the literature reports, perticularly in the case of PGE₁ . It is documented[14,15] that in isolated adipose tissues PGE₁ stimulates glucose uptake.

Cardiac output is generally increased by the prostaglandins of E and F series [16]. It is also known[17] that adrenaline is a powerful cardiac stimulant and enhances cardiac output by acting on β_1 receptors. The data obtained in the present study (Table III) indicate that transport of adrenaline

TABLE III

Solute Permeability (ω)[a] of Various Permeants in Presence of Liquid Membranes Generated by Prostaglandin E_1 (ω_1) and Prostaglandin F_2 (ω_2) in Lecithin-Cholesterol Mixtures[b] along with the Control Values (ω_0) When no Prostaglandin was Used.

Permeants	Intial Concentration (mg liter^{-1})	$\omega_0 \times 10^9$ (mole s^{-1}N^{-1})	$\omega_1^c \times 10^9$ (mole s^{-1}N^{-1})	$\omega_2^d \times 10^9$ (mole s^{-1}N^{-1})
Glucose	10	0.288±0.030	0.412±0.070	0.360±0.011
Histamine	10	0.392±0.019	0.244±0.007	0.229±0.001
Adrenaline	100	1.592±0.038	1.697±0.026	2.216±0.009
Ethinyl estradiol	30	2.280±0.046	3.135±0.035	3.424±0.068
Progesterone	100	0.198±0.032	0.697±0.093	0.279±0.006
Glycine	100	1.517±0.061	2.284±0.059	1.279±0.034
γ-Amino butyric acid	200	0.916±0.012	1.170±0.024	1.153±0.039
Sodium chloride	5.382	0.133±0.008	0.196±0.012	0.197±0.002
Potassium chloride	10.430	0.122±0.008	0.055±0.001	0.101±0.003

[a] Values of ω are reported as arithmetic means of 15 repeats ± SD.

[b] Lecithin concentration 15.542 ppm; cholesterol concentration, 1.175×10^{-6} M.

[c] Prostaglandin E_1 concentration, 0.65×10^{-8} M.

[d] Prostaglandin $F_{2\alpha}$ concentration, 8.5×10^{-8} M.

is increased in the presence of the liquid membranes generated by the prostaglandins. This observation suggested that increased permeability of adrenaline due to the prostaglandins present in the membranes of myocardial cells facilitating interaction with β_1 receptor may also be a contributing factor to the reported increase in cardiac output by the prostaglandins.

Prostaglandins of E series are known to inhibit the gastric acids secretion stimulated by feeding histamine[18-19], and this has raised the possibility of the therapeutic utility of certain methylated analogs of prostaglandins for peptic ulcers[20]. The gastric acid secretion by histamine is exerted through H_2 receptors and is blocked by H_2 receptor antagonists[21]. It has been indicated recently[22] that an impediment in the transport of histamine due to the liquid membrane which are likely to be generated by the H_2 receptor antagonist drugs like cimetidine and ranitidine at the site of action may also contribute to their H_2 - antagonistic action. The present data on the transport of histamine in the presence of PGE_1 liquid membrane (Table III) appear relevant to the reported[18,19] inhibition of gastric acid secretion by the PGE_1. It appears that the resistance offered by

the PGE_1 to the transport of histamine, impeding its acces to the H_2 receptors, may also be a cause of the inhibition of histamine - induced gastric acid secretion from the parietal cells. Although histamine transport is also impeded in the presence of $PGF_{2\alpha}$ (Table III), the relevance of this observation in the context of gastric acid secretion is not clear .

Table III reveals that in the case of PGE_1 the transport of both glycine and GABA is enhanced where as in the case of $PGF_{2\alpha}$ the transport of GABA is enhanced and that of glycine is impeded. The enhancement in the transport of glycine and /or GABA leading to their increased concentration in the brain could also be the reason for reported [23-25] effects such as sedation, stupor, catatonia, etc., induced by the administration of prostaglandins, particularly PGE_1, in animals.

It is reported [23] that in the intact central nervous system of a chloralose- aneasthetized chick, intravenous administration of $PGF_{2\alpha}$ potentiates the crossed extensor reflex while PGE_1 inhibits it. The opposing trends observed in the transport of glycine (Table III), which is known [26] to be utilized by the inhibitory interneurons of the spinal cord, may be

relevant to the reported potentiation and inhibition of the crossed extensor reflex in chicks.

Prostaglandins have been used as abortive agents[27]. The present data on the permeability of estrogen and progesterone (Table III) appear relevant to their abortive actions. Not only high concentrations of estrogen and progesterone but also a proper ratio of their concentrations are essential for the maintenance of pregnancy[28]. As the present data indicate the presence of high concentration of prostaglandins in the membranes of the uterus would enhance the outflow of estrogen and progesterone to an *unequal* equal extent. This outflow would not only decrease the concentrations of estrogen and progesterone but also disturb the estrogen-progesterone ratio resulting in the failure of pregnancy.

The present data on the permeability of estrogen and progesterone also appear relevant to the causation of primary dysmenorrhea. There is substantial evidence to indicate that prostaglandin is a major causal factor in primary dysmenorrhea[29]. Drugs having prostaglandin synthetase inhibitory activity have been reported to be effective in the

treatment of dysmenorrhea. The effectiveness of oral
contraceptives in the treatment of dysmenorrhea is
also well established[29]. These observations appear
to indicate that the enhanced permeability (outflow)
of estrogen and progesterone in the presence of the
increased concentration of prostaglandins, particularly
$PGF_{2\alpha}$, in the endometrium may also be a factor respo-
nsible for dysmenorrhea.

Prostaglandins of E and F series are present
in the renal medulla. Renal prostaglandins have been
implicated in antihypertensive action[30]. It is
suggested that prostaglandins may exert an antihy-
pertensive action acting either as peripheral vasodila-
tors or by promoting diuresis with sodium loss, i.e.,
natriuresis[30]. The enhanced permeability to sodium
ions in the presence of prostaglandins as observed
in the present experiment appear consistent with the
latter mechanism. Sodium reabsorption in proximal
tubule is active in nature and is mediated by carbonic
anhydrase[31]. In addition to forces moving sodium
ion and water out of the proximal tubule there is a
component of leakage back across the tubular epithe-
lium into the lumen of the proximal nephron[32]. The

back leak is passive in nature and its amount is influenced by peritubular osmotic pressure[32]. The increased passive transport of sodium ions in the presence of prostaglandins (Table III) may thus offer an explanation for the diuretic and natriuretic effects of the prostaglandins due to the back-leak mechanism leading to their antihypertensive action.

The toxin vibrio/cholerae affects electrolyte handling by the epithelial cells of the intestinal mucosa in such a way that there is hyper secretion into the gut resulting in the profuse watery stools that characterise Cholera. It has been suggested[33] that the toxin acts by stimulating prostaglandin synthesis. The enhanced permeability of sodium ions in the presence of prostaglandins as observed in the present study (Table III) suggests that a back-leak mechanism similar to the one proposed in the case of the natriuretic and diuretic effects of the prostaglandins[32] may also explain the hyper secretion into the lumen of the intestines due to the increased concentration of the prostaglandin in the epithelial cells of the intestinal mucosa.

$PGF_{2\alpha}$ does not affect the transport of potassium ions significantly (Table III). In the presence of PGE_1, however, a decrease in the transport of potassium ions is observed (Table III). The observation on the decreased permeability of potassium ions may be relevant to the causation of Bartter's syndrome. Bartter's syndrome, an unusual and complex disorder which is characterized by among other symptoms, hypokalemia, i.e., excessive loss of potassium has been associated with excessive production of renal prostaglandis[34]. This is obvious from the fact that Bartter's syndrome has been successfully treated with drugs like indomethacin and aspirin [35-38] which have prostaglandin synthetase inhibitory activity. Although potassium reabsorption in proximal tubules is active in nature, the present data suggest that impediments in the transport of potassium ion due to the increased concentration of the prostaglandin in the tubular cells may also contribute to the urinary potassium wasting leading to hypokalemia.

The present study thus indicates that the phenomenon of liquid membrane formation may play a significant role in the biological action of the prostaglandins.

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CHAPTER V

LIQUID MEMBRANE PHENOMENA IN ANTICANCER
DRUGS STUDIES ON 5-FLUOROURACIL AND ITS
DERIVATIVES

CHAPTER V

LIQUID MEMBRANE PHENOMENA IN ANTICANCER DRUGS-
STUDIES ON 5-FLUOROURACIL AND ITS DERIVATIVES*

One of the important implications of the liquid membrane hypothesis of drug action[1] (Chapter I) is that in a series of structurally related drugs which are congeners of a common chemical moiety and which act by altering the permeability of cell membranes, any structural variation which increases the hydrophobicity of the compound will increase the potency of the drug while any alteration of the hydrophilic moieties of the drug may change the nature of its action qualitatively[1].

It has been shown by Iigo et.al[2], that the newly synthesized[3] 1-hexylcarbamoyl-5-fluorouracil (HCFU) is more active against various tumours in mice and less

*Based on the paper published in the International Journal of Pharmaceutics, 38, 239-245 (1987).

toxic to host animals than its parent drug 5-fluorouracil (5FU). Iigo et al[2] have tested the activities of these drugs on Lewis lung carcinoma and B16 melanoma. It is evident from the structure of two drugs (Fig.1) that HCFU will be more hydrophobic and more surface active than its parent compound 5FU prompted by this clue, 5FU and two of its derivatives, HCFU and 1-(2-tetrahydrofuryl) 5-fluorouracil (FT), have been investigated for the contribution of liquid membrane phenomena to their action, the results are reported in this Chapter.

All the 3 drugs, 5FU, HCFU and FT, have been shown to generate liquid membranes in series with a supporting membrane. Transport of relevant permeants through the liquid membranes generated by these drugs in series with the supporting membrane has been studied. The data obtained from these model experiments indicate that the modification in the transport of relevant permeants due to the drug liquid membranes likely to be generated at the sites of action may also make a significant contribution to the biological actions of these drugs. In these studies a non-specific non-living membrane has been chosen deliberately as the
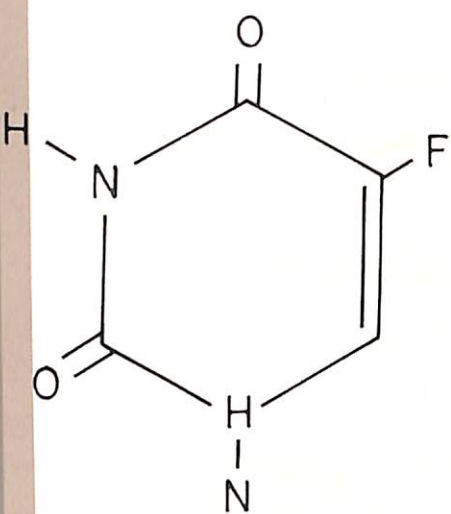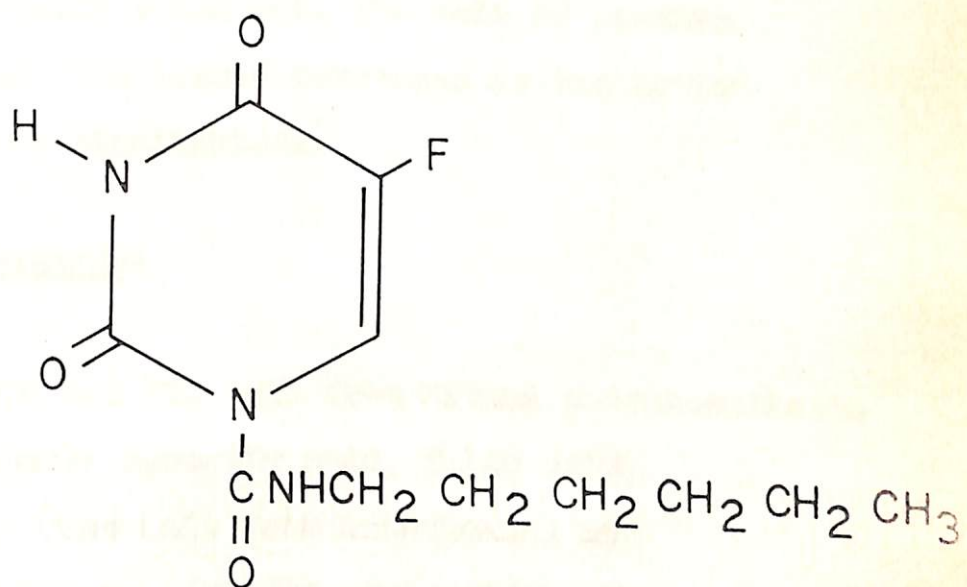
(a)   (b)

Fig. 1 : Chemical structures of (a) 5-Fluorouracil and
          (b) 1-hexylcarbamoyl -5 Fluorouracil.

supporting membrane for the liquid membranes. Thus the possibility of active and specific interaction of these drugs with the constituents of biomembranes as a cause for modification in the transport of relevant permeants is totally ruled out. The role of passive transport through the liquid membranes in the action of these drugs is highlighted.

Materials and Methods:

Materials:

HCFU, 5FU and FT, (all from Mitsui pharmaceuticals Inc., Tokyo, Japan): aspartic acid, folic acid, glutamine and glycine (all from Lobachemie) and cyanocabalamine (Sigma V-2876) were used in the present experiments. All other chemicals were analytical grade reagents. Distilled water, distilled twice in an all-pyrex glass still, was used for preparing the solutions. Aqueous solutions of HCFU and FT, which are not so easily soluble in water, were prepared by adding to the aqueous phase, with vigorous stirring, the necessary volume of ethanolic stock solution of known concentration of these substances. In the aqueous solutions of HCFU and FT thus prepared the final concentration of ethanol was never allowed to

exceed 0.1% (v/v). It was experimentally found that 0.1% solution of ethanol in water did not lower the surface tension of water to any measurable extent.

Methods :

The critical micelle concentrations (CMCs) of 5FU, HCFU and FT were estimated from the variation of surface tension with concentration and are recorded in Table 1. The surface tensions were measured using a Fisher tensiomat - model 21.

The all glass cell described in Chapter II was used for the transport studies. A sartorius cellulose acetate membrane (Cat. no. 11107 , pore size 0.2 um) of thickness 1×10^{-4} M and area 2.55×10^{-5} m^2 which acted as supporting membrane for the liquid membrane separated the transport cell into two compartments C and D (Fig. 1 of Chapter II). To obtain the hydraulic permeability data which were utilised to demonstrate the formation of liquid membrane in series with the supporting membrane, aqueous solutions of varying concentrations of the drugs were filled in compartment C of the transport cell (Fig.1 of Chapter II) and compartment D was filled with distilled water.

The concentrations of the drugs chosen were such that the hydraulic permeability data were obtained both below and above the CMCs of the drugs. The details of the method used for the hydraulic permeability measurements have been described in Chapter II.

The solute permeability (ω) of the relevant permeants in the presence of the liquid membranes generated by the drugs was estimated using the method described in Chapter II. For solute permeability measurements, two sets of experiments were performed. In the first set of experiments an aqueous solution of the drug was filled in compartment C along with the permeant and compartment D was filled with distilled water (Fig. 1 Chapter II). In the second set of experiments the aqueous solution of the drug was filled in compartment D of the transport cell and compartment C was filled with the aqueous solution of the permeant. The concentrations of the drugs used in the ω measurements were always higher than their CMCs.

All measurements were made at constant temperature using a thermostat set at 37 $\pm$ 0.1°C.

Estimations

The amounts of various permeants transported to compartment D were estimated as follows:

Folic Acid: The amount of folic acid was estimated using a Cary 17-D spectrophotometer by the measurement of ultraviolet absorption at 283 nm - the absorption maximum of folic acid in 0.1 M sodium hydroxide solution.[4]

Cyanocobalamin: The amount of cyanocobalamin was estimated spectrophotometrically in an aqueous solution by measurement of absorbance at 361 nm[4].

Amino Acids: The amounts of glycine, glutamine and aspartic acid were estimated from the amount of their reaction products with ninhydrin measured at 570 nm[5].

Results and Discussion:

The hydraulic permeability data at various drug concentrations in the case of all three drugs, were found to be in accordance with the proportional relationship

$$J_v = L_p \, \Delta P \qquad (1)$$

where J_v represents the volume flux per unit area of the membrane, L_p the hydraulic conductivity coefficient and ΔP the applied pressure difference across the membrane. The values of L_p estimated from the slopes of J_v versus Δp plots, in the case of all 3 drugs, show a progressive decrease with increase in the concentrations of the drugs (Table II) upto the CMCs of the drugs beyond which they become more or less constant. This trend in the values of L_p is consistent with the Kesting's hypothesis[6] and as discussed in Chapter II indicative of formation of a liquid membrane in series with the supporting membrane. Analysis of the value of L_p in the light of the mosaic model[7,8,9] furnishes additional evidence in favour of the formation of liquid membranes by the drugs in series with the supporting membrane. Following the arguments of progressive coverages, as shown in Chapter II, the values of L_p at concentration equal to or less than the CMC of the surface-active drug should be equal to $(1-n)L_p^s + n L_p^c$ where $n \leq 1$ and the superscripts s and c represent the values of L_p at 0 and CMC of the surfactant respectively. The values of L_p thus computed at several concentrations of the drugs below their CMCs compare favourably with corresponding experimental values in the case of all the 3 fluorouracils (Table II).

TABLE II

Values of L_p at various Concentrations of 5FU, FT and HCFU

	Conc. $(\times 10^{11}\ M)$	$L_p \times 10^8$ $(m^3 s^{-1} N^{-1})$*	$L_p \times 10^8$ $(m^3 s^{-1} N^{-1})$**
5FU	0.000	-	2.162 ± 0.064
	20.00(0.25CMC)	1.930 ± 0.058	1.944 ± 0.056
	40.00(0.5CMC)	1.720 ± 0.054	1.726 ± 0.059
	60.00(0.75CMC)	1.573 ± 0.074	1.508 ± 0.041
	80.00(CMC)	1.290 ± 0.034	-
	160.00	1.260 ± 0.061	-
	240.00	1.266 ± 0.064	
FT	0.000	2.162 ± 0.064	-
	1.875(0.25CMC)	1.778 ± 0.086	1.805 ± 0.081
	3.750(0.5CMC)	1.418 ± 0.049	1.406 ± 0.115
	5.625(0.75CMC)	1.095 ± 0.059	1.106 ± 0.039
	7.500(CMC)	0.755 ± 0.017	-
	15.00	0.751 ± 0.025	-
	22.500	0.761 ± 0.031	-
HCFU	0.000	2.162 ± 0.064	-
	1.525(0.25CMC)	1.795 ± 0.041	1.770 ± 0.049
	3.050(0.5CMC)	1.422 ± 0.030	1.377 ± 0.036
	4.575(0.75CMC)	0.999 ± 0.018	0.985 ± 0.023
	6.100(CMC)	0.592 ± 0.010	-
	12.200	0.594 ± 0.006	-
	18.300	0.582 ± 0.018	-

The values reported for L_p are arithmetic mean of 10 repeats
$\pm$ S.D.

* Experimental values.
**Calculated values using mosaic model

Solute Permeability Data:

Since all 3 drugs, being surface active in
nature, have both hydrophilic and hydrophobic parts in
their structure, it is expected that the hydrophobic
ends of the drugs molecules in the liquid membrane would
be preferentially oriented towards the hydrophobic
supporting membrane and the hydrophilic moieties would
be drawn outwards away from it. Thus in the first set
of solute permeability experiments the permeants would
face the hydrophilic surface of the drug - liquid
membrane generated in series with the supporting
membrane while in the second set of experiments they
would face the hydrophobic surface. The data on the
solute permeability of relevant permeants in the two
orientations of the drug molecules in the liquid mem-
branes are recorded in Table II along with the correspond-
ing values from control experiments where no drug was
used.

Antimetabolites in general are known to act by
impairing the synthesis of purine and pyrimidine bases
by interfering with folic acid metabolism or prevent
the incorporation of the bases into nucleic acids[10].
The steps involved are known to be enzyme-catalysed.
For example 5 FU is ultimately converted enzymatically

TABLE III

Solute permeability (ω) of Various Permeants in the Presence of 5FU, FT and HCFU

Permeant	Initial concentration (mg/litre)	Control $\omega \times 10^9$	5FU(1 x 10^{-9} M)		FT(1 x 10^{-10} M)		HCFU(1 x 10^{-10} M)	
			$\omega \times 10^9$	$\omega \times 10^9$	$\omega \times 10^9$	$\omega \times 10^9$	$\omega \times 10^9$	$\omega \times 10^9$
			C	D	C	D	C	D
Aspartic acid	150	0.856 ±0.011	0.628 ±0.004	0.688 ±0.006	0.475 ±0.020	0.715 ±0.062	0.398 ±0.008	0.568 ±0.042
Cyanocobalamin	30	0.488 ±0.018	0.281 ±0.024	0.365 ±0.021	0.316 ±0.015	00.379 ±0.018	0.282 ±0.026	0.347 ±0.037
Folic acid	0.05	8.715 ±0.266	6.013 ±0.557	7.541 ±0.316	6.631 ±0.496	7.590 ±0.010	4.406 ±0.220	5.743 ±0.3
Glutamine	500	0.474 ±0.031	0.759 ±0.065	0.694 ±0.052	0.160 ±0.011	0.363 ±0.014	0.417 ±0.013	0.399 ±0.008
Glycine	100	0.265 ±0.010	0.412 ±0.055	0.644 ±0.168	0.151 ±0.002	0.182 ±0.003	0.181 ±0.001	0.195 ±0.004

Values of ω are reported as arithmetic mean of 10 repeats ± S.D., in mol. $Ss^{-1} N^{-1}$, C, drug in Compartment C; D, drug in compartment D.

into 5-fluorodeoxyuridine-5-phosphate which inhibits the thymidylate synthetase enzyme system resulting in the blockade of DNA synthesis [11]. The present data (Table III), however, indicate that the passive transport through the liquid membranes likely to be generated by the fluorouracils (5-FU, HCFU and FT) at the respective sites of action may also contribute to their action.

Vitamin B_{12} and folic acid, which are dietary essentials for man, are required for the synthesis of purine and pyrimidine bases and their incorporation into DNA. Their deficiency may result in defective synthesis of DNA in any cell that attempts chromosomal replication and division [12]. The present data indicate that the transport of both vitamin B_{12} and folic acid are impeded by the liquid membranes generated by all 3 drugs presently studied (Table II). This impediment in the transport may contribute to the deficiency of vitamin B_{12} and folic acid inside the cells resulting in the defective synthesis of DNA. Thus it appears that the phenomena of liquid membrane formation may also contribute to the anticancer activities of 5FU and its derivatives. A perusal of Table II further reveals that inhibition in the transport of
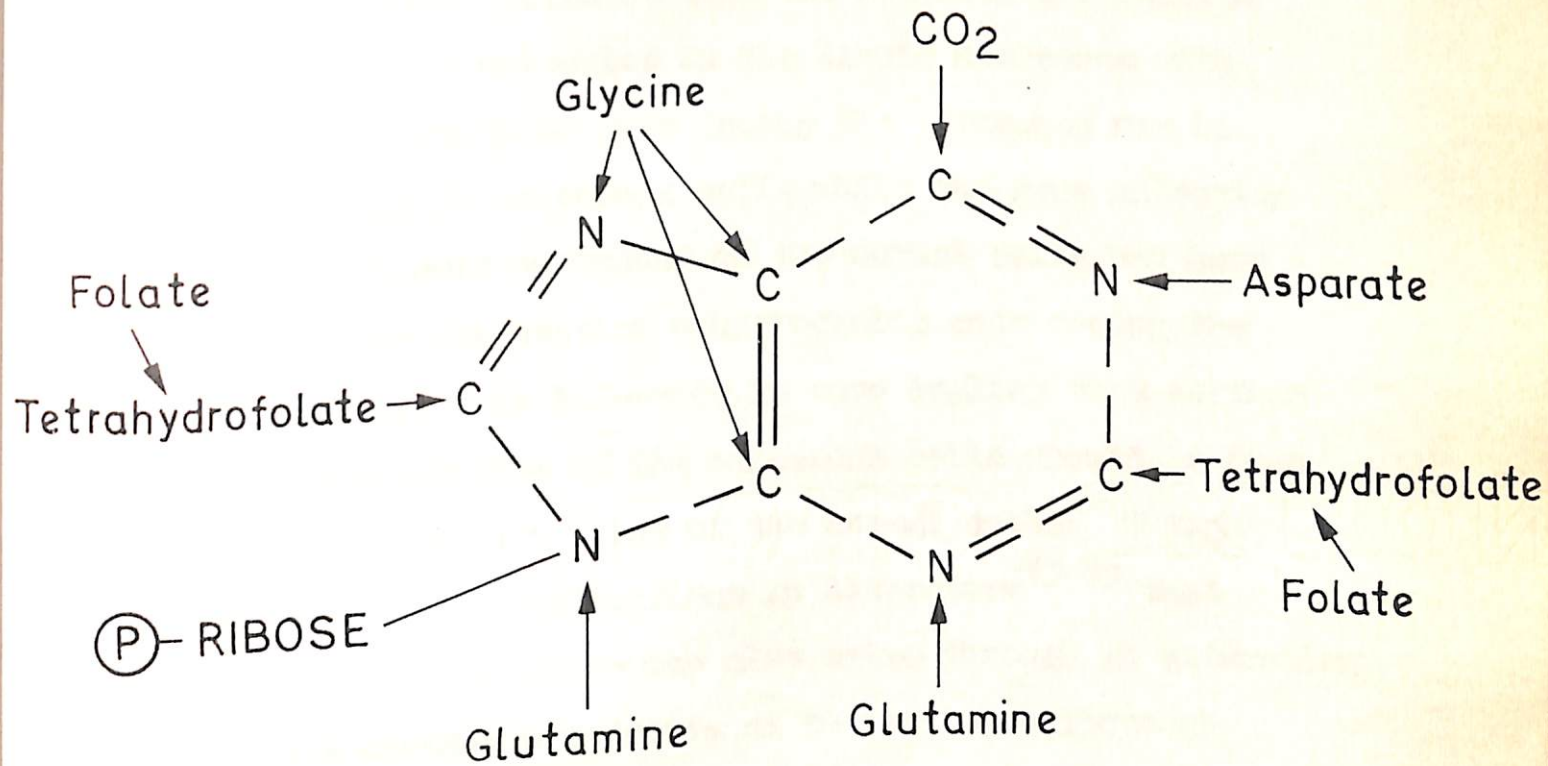
Fig.2 : Compounds from which the atoms of the purine ring
are derived in the biosynthetic pathway. The breaks
in the bonds separate the groups of atoms derived
from each source.

vitamin B_{12} and folic acid, is more when the permeants face the hydrophilic surface of the liquid membranes than when they face the hydrophobic surface. This observation indicates that the specific orientation of the drug molecules in the liquid membranes with their hydrophilic ends facing the permeants may be necessary on cancerous cells while the drug molecules in the liquid membranes on the normal cells may have the other orientation - hydrophobic ends facing the permeants. This inference in turn implies that surface of the membranes of the cancerous cells should be less hydrophilic than those of the normal cells. Though there are some indications in literature[13-15] that the neoplastic state may also arise through an alteration in the surface properties of the cells, a thorough probe in terms of hydrophilicity of the cell surface is called for to substantiate this conjecture.

Amino acids like glycine, glutamine and aspartic acid are also required, in addition to folic acid, for the purine ring synthesis[16,17]. Compounds from which the atoms of the purine ring are derived in the biosynthetic pathway are depicted inFig.2. The present data (Table 3) indicate that except in the case of 5FU , the transport of glycine, glutamine and aspartic

acid is also impeded in addition to folic acid
and vitamin B_{12} by the liquid membranes generated by
both FT and HCFU. In the case of 5FU the transport
of glycine and glutamine was enhanced. The impediment
in the transport of the amino acids also in the case
of HCFU and FT was more in the specific orientation of
the drug molecules in the liquid membrane with their
hydrophilic ends facing the permeants. This impediment
in the transport observed in the case of FT and HCFU
may also be a factor responsible for the impairment
of the synthesis of purine bases contributing to the
anticancer activity of these drugs.

It has been shown by Iigo et.al.[2] that of the
3 drugs, HCFU, FT and 5FU, HCFU is most potent. This
finding is most consistent with the liquid membrane
hypothesis for drug action[18]. The CMC of HCFU is the
lowest (Table I). As CMC is the concentration at which
a complete liquid membrane is generated at the inter-
face, it would appear that of the three drugs, HCFU
would require the lowest concentration for the deve-
lopment of a complete liquid membrane at the site of
action. Since modification in the transport of the
relevant permeants, which affects the biological effect
is maximum when a complete liquid membrane is generated,

the concentration of HCFU required to produce the maximum biological effect would be the lowest amongst the 3 drugs, making HCFU the most potent drug.

Some of the adverse side effects of cytotoxic drugs include megaloblastic anaemia[19], neurological disorders relating to spinal column and cerebral cortex[20], ineffective haematopoiesis and pancytopenia[21]. These symptoms are also the symptoms of deficiencies of vitamin B_{12} or folic acid or both [22-25]. The impediment in the transport of B_{12} and folic acid in the specific orientation of the drug molecules in the liquid membrane with their hydrophobic ends facing the permeants, which may be the orientation on the normal cells, could also be a plausible explanation for the reported side-effects.

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CHAPTER VI

LIQUID MEMBRANE PHENOMENON IN VITAMIN E
STUDIES ON α-TOCOPHEROL.

CHAPTER VI

LIQUID MEMBRANE PHENOMENON IN VITAMIN E:
STUDIES ON ∝-TOCOPHEROL*

∝-Tocopherol is the most important tocopherol because it comprises about 90% of the tocopherols in animal tissues and exhibits maximum biological activity. It is distributed throughout the tissues of animals and man and its deficicney causes a variety of syndromes in the animal organism. Just by looking at the structure of ∝-tocopherol one suspects it to be surface-active in nature. Since according to Kesting's hypothesis[1], surface active agents, when added to aqueous phase, generate a surfactant layer liquid mambrane at the interface, it is likely that the phenomenon of liquid membrane formation may play a role in the actions of ∝-tocopherol.

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In the present study investigations were carried out to explore the role of liquid membrane phenomenon in the actions of α-tocopherol. Critical micelle concentration of α-tocopherol in water has been determined. The data on hydraulic permeability have been obtained to demonstrate: (1) the formation of a liquid membrane by α-tocopherol in series with the supporting membrane: and (ii) the incorporation of α-tocopherol in the lecithin-cholesterol liquid membrane existing in series with the supporting membrane. Transport of relevant permeants in the presence of the liquid membrane generated by the lecithin-cholesterol-α-tocopherol mixture has been studied and the data obtained have been discussed in the light of the various syndromes caused by vitamin E deficiency.

Materials and Methods:

MATERIALS:

Lecithin (egg phosphatidyl choline) and progesterone (Patel chest Institute, CSIR Centre for Biochemical, Delhi), Cholesterol (Centron Research Laboratories , Bombay). DL -α-tocopherol acetate (Sigma T 3376), eththyloestradiol (Roussel pharmaceuticals (India) Bombay.Cystine, methionine and creatinine (all from Loba Chemie), sodium, potassium and calcium

chlorides (all Analar grade) and distilled water distilled twice in an all-pyrex glass still were used in the present experiments.

METHODS

Aqueous solutions of desired concentration of α-tocopherol, lecithin-cholesterol α-tocopherol mixture ethinyl oestradiol and progesterone which are not so easily soluble in water were prepared by adding necessary volume of ethanolic stock solution of known concentration to aqueous phase with constant stirring. In the aqueous solutions thus prepared the final concentration of ethanol was never allowed to exceed 0.1% by volume because it was experimentally shown that a 0.1% solution of ethanol in water did not lower the surface tension of water to any measurable extent. The aqueous solutions of cystine, methionine, creatinine and chlorides of sodium, potassium and calcium were prepared in the usual way.

The critical micelle concentration (CMC) of aqueous α-tocopherol, aqueous lecithin and aqueous cholesterol were determined from the variation of their surface tension with concentration and were found to be

5.0×10^{-8}M, 1.599×10^{-5}M and 30.08×10^{-9}M, respectively. Surface tensions were measured using a Fisher tensiomat model 21. The all glass cell described in Chapter II were used for the transport studies . A Sartorius cellulose acetate microfiltration membrane (Cat. no. 11107, pore size 0.2 um) of thickness 1×10^{-4}M and area 2.55×10^{-5}m^2 which infact acted as a supporting membrane for the liquid membranes, devided the transport cell into two compartments C and D. (Fig. Chapter II) To obtain the hydraulic permeability data, the solutions of varying concentrations of α-tocopherol in water or in the aqueous solution of the lecithin -cholesterol mixture of fixed composition were filled in compartment C of the transport cell and the compartment D was filled with water, (Fig. 1 of Chapter II). The details of the methods used for the hydraulic permeability measurments have been described earlier in Chapter II.

For solute permeability (ω) measurement various permeants namely estrogen, progesterone, cystine, methionine, creatinine, and sodium, potassium and calcium ions, the procedure described earlier as in Chapter II was followed Compartment C of the transport cell was filled with the solution of known concentration of the permeant prepared in the aqueous

solution of lecithin, cholesterol and α-tocopherol
mixture of composition 1.919×10^{-5} M with respect to
lecithin, 1.175×10^{-6} M with respect to cholesterol
and 3.75×10^{-8} M with respect to α-tocopherol and
compartment D was filled with distilled water. In
control experiments, however, no α-tocopherol was
used.

This particular composition of the aqueous
solution of lecithin - cholesterol - α-tocopherol
mixture, used in the experiments for solute permeability
measurement which was derived from our earlier study[2,3]
and the present data on hydraulic permeability, is the
composition at which the lecithin liquid membrane com-
pletely covers the supporting membrane and is saturated
with both cholesterol and α-tocopherol. Since
lecithin, cholesterol and α-tocopherol are all
surface active in nature and have both hydrophobic
and hydrophilic parts in their structure, it is obvious
that in the liquid membrane generated in these
experiments the hydrophobic tails of these molecules
will be preferentially oriented towards the hydrophobic
supporting membrane and the hydrophilic moieties would
be drawn outwards away from it.

The value of the solute permeability (ω)
were estimated using method described in Chapter II.

All the measurements were carried out at con-
stant temperature using a thermostat set at $37 \pm 0.1°C$

Estimations:

The amounts of various permeants transported
to compartment D were estimated as follows:

(1) Amino Acids:

The amounts of cystine and methionine were
estimated from the amount of their reaction products
with nin-hydrin measured at 570 nm [4] using a Bausch
and Lomb spectronic -20 spectrophotometer.

(2) Creatinine:

The amount of creatinine estimated using the
method described in literature [5] in which creatinine
was subjected to react with alkaline picrate to form
an orange colored Jaffe complex', the intensity of
which was measured spectrophotometrically at 520 nm.

(3) <u>Oestrogen</u> :

The amount of ethinyl oestradiol was estimated by a chemical method, [6] using quinol - H_2SO_4 as a coloring reagent and the color developed was measured at 538 nm using a spectrophotometer.

(4) <u>Progesterone</u>:

The amount of progesterone is estimated using a Cary 17-D spectrophotometer at 242 nm the absorption maximum or progesterone[7].

(5) <u>Cations</u> :

The amount of sodium, potassium, and calcium ions were determined using a flame photometer C model CL-22 Elico-India).

<u>Results and Discussion</u>:

The hydraulic permeability data at various concentration of α-tocopherol where in all cases, found to be represented by the equation:

$$J_v = L_p \cdot \Delta P \qquad (2)$$

where J_v represents the volume flux p r unit area of the membrane, ΔP applied pressure difference and L_p stands for the hydraulic conductivity coefficient. The

values of L_p at various concentrations of α-tocopherol, estimated from the slopes of the J_v versus ΔP plots show a decreasing trend with the increase in concentration of α-tocopherol upto its CMC beyond which they become more or less constants. (Table I). This trend indicates progressive coverage of the supporting membrane with the liquid membrane generated by α-tocopherol in accordance with the Kesting's hypothesis[7]. At the CMC the α-tocopherol liquid membrane completely covers the supporting membrane. Analysis of the transport data (Table I) in light of mosaic membrane model furnishes[8-10] further support infavour of the formation of liquid membrane in series with the supporting membrane.

Information about the incorporation of α-tocopherol in the liquid membrane generated at the interface by lecithin-cholesterol mixture can be gathered from the hydraulic permeability data for solutions of various concentrations of α-tocopherol prepared in the aqueous solutions of lecithin- cholesterol mixtures of fixed composition, i.e., 1.919×10^{-5} M with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol, which infact is the composition at

TABLE - I

Values of L_p at Various Concentrations of α-Tocopherol

Concentration $\times 10^8$ M	0.00	1.25 (0.25CMC)	2.50 (0.5CMC)	3.75 (0.75CMC)	5.00 (1 CMC)	10.00	15.00
$L_p^a \times 10^8$ $(m^3.s.^{-1}N.^{-1})$	4.891 ± 0.137	4.473 ± 0.189	4.078 ± 0.121	3.669 ± 0.113	3.170 ± 0.077	3.112 ± 0.141	3.098 ±0.042
$L_p^b \times 10^8$ $(m.^3s.^{-1}N.^{-1})$	-	4.461 ± 0.122	4.031 ± 0.107	3.600 ± 0.092			

a Experimental values

b Calculated values using mosaic model

which the liquid membrane generated by lecithin completely
covers interface and is saturated with cholesterol[3].
The values of L_p at various concentration of α-toco-
pherol estimated, from the J_v versus ΔP plots, which
in this case also were found to be in accordance
with equation (1) are recorded in Table II. The decreasing
trend in the values of L_p which continues upto the
α - tocopherol concentration equal to 3.75×10^{-8}M
(Table II) indicates that more and more of α-tocopherol
is incorporated in the lecithin-cholesterol liquid
membrane generated at the interface and at concentration
equal to 3.75×10^{-8}M the lecithin cholesterol liquid
membrane is saturated with α-tocopherol. In order to
ascertain whether the added α-tocopherol reaches straight
up to the interface or not, surface tensions of
solutions of various concentrations of α-tocopherol
prepared in the aqueous solution of the lecithin-
cholesterol mixture of composition 1.919×10^{-5}M with
respect to lecithin and 1.175×10^{-6}M with respect to
cholesterol, were measured. The surface tension of
the aqueous solution of the lecithin-cholesterol mixture
showed a further decrease on addition of α-tocopherol
and the decreasing trend continued upto the α-tocopherol
concentration equal to 3.75×10^{-8}M. This trend

TABLE - II

Values of L_p at Various Concentrations of —Tocopherol in Lecithin-Cholesterol- —Tocopherol Mixtures[a]

Concentration x 10^8 M	0.00	1.25	2.50	3.75	5.00	10.00
L_p x 10^8 (m^3.s^{-1}N^{-1})	1.575±0.084	1.402±0.045	1.296±0.012	1.178±0.011	1.185±0.031	1.164±0.033

[a] Lecithin and cholesterol concentrations kept constant at 1.919×10^{-5} M and 1.175×10^{-6} M, respectively.

indicates that the added α-tocopherol reached deep up to the interface in the liquid membrane generated by the lecithin-cholesterol mixture in series with the supporting membrane.

Solute permeability data:

Data on the solute permeability (ω) of several permeants, namely oestrogen, progesterone, cystine, methionine, creatinine and cations (Na^+, k^+ and Ca^{++} ions), in the presence of liquid membrane generated by the mixture of lecithin, cholesterol and α-tocopherol in series with the supporting membrane are recorded in Table III. The data appear to be relevant to causation of various syndromes in animal organisms due to deficiency of vitamin E i.e., α-tocopherol.

Except for the work cited by Wagner and Folkers[11] there is enough evidence to indicate that vitamin E is essential for normal reproduction in several mammalian[12,13] species and its deficiency is known to cause habitual abortions. The fundamental mechanism by which vitamin E deficiency interferes with reproduction is obscure[13]. The present data (Table III) on the impediments in the transport of oestrogen and progesterone

TABLE - III

Solute Permeability (ω) of Various Permeants in Presence
of Lecithin-Cholesterol - α -Tocopherol Mixture[a]

	$\omega^b \times 10^9$ (mol.s^{-1}N^{-1})	$\omega^c \times 10^9$ (mol.s^{-1}N^{-1})
Methionine[d]	8.79±0.27	6.25±0.24
Cystine[e]	2.62±0.09	4.66±0.56
Creatinine[f]	0.27±0.03	0.29±0.02
Ethinyl oestradiol[g]	5.50±0.40	4.14±0.24
Progesterone[h]	4.90±0.38	4.11±0.20
Sodium(chloride)[i]	0.12±0.01	0.12±0.01
Potassium(chloride)[j]	0.13±0.01	0.14±0.01
Calcium(chloride)[k]	0.16±0.03	0.18±0.01

[a] Lecithin concentration, 1.919 x 10^{-5} M; cholesterol concentration, 1.175 x 10^{-6} M; α -tocopherol concentration, 3.75 x 10^{-8} M.

[b] Control value when no α -tocopherol was used.

[c] Lecithin-cholesterol- α -tocopherol mixture in compartment C together with the permeant.

[d] Initial concentration 100 mg/L

[e] Initial concentration 100 mg/L

[f] Initial concentration 1g/L

[g] Initial concentration 50 mg/L

[h] Initial concentration 100 mg/L

[i] Initial concentration 5.382 g/L

[j] Initial concentration 10.430 g/L

[k] Initial concentration 0.222 g/L

in the presence of α-tocopherol may offer an explana-
tion for occurence of habitual abortions caused by
vitamin E deficiency.

It is not only the high concentration of
oestrogen and progesterone but also a proper ratio of
their concentration which is essential for the mainte-
nance of pregnancy[14]. As the present data indicate
deficiency of vitamin E in the membranes of the uterus
would enhance the outflow of oestrogen and progesterone
to an unequal extent. This outflow would disturb the
oestrogen - progesteron ratio resulting in the failure
of pregnancy.

In many species, deficiency of vitamin E leads
to the development of muscular dystrophy. Metabolic
disturbances during muscular dystrophy include increased
water content, changes in electrolyte pattern and
increased excretion of creatine in urine-creatinurea[15].
The values of solute permeability (ω) for the cations
and also for creatinin in the presence of α-tocopherol
do not show any significant difference in comparison
to the values obtained from the control experiment where

no α -tocopherol was used. The data on hydraulic permeability (Table 2), however, appear relevant to causation of increased water content of the tissues and creatinurea. The data in Table 2 imply that the cell membranes deficient in vitamin E are likely to be more permeable to water which may be one of the factors responsible for the increased water content of the tissue. It has been suggested [15,16] that creatinurea in nutritional muscular dystrophy might be due to hydration of creatinine to creatine due to increased water content of the tissues-creatinine is formed inside the cells as a result of creatine metabolism. The alteration in the normal water balance of tissues is a consistent finding in the biochemical and histological examination of tissues affected by vitamin E depletion[15]. Nitowsky[17] et.al. have shown that tocopherol can decrease the elevated creatine excretions of children with cystic fibrosis.

Dam and associates[18] have shown that muscular dystrophy in chicks could be prevented by supplementing the diet with either vitamin E or cystine. Later Machlin and Shalkop[19] showed that cystine and methionine

were equally effective in prevention of dystrophy. However, Scott and Calvert[20] have reported that cystine is more effective than methionine. The present data (Table 3) show that the permeability of cystine is enhanced and the amount of methionine was reduced in the presence of α-tocopherol. This observation is consistent with the inference drawn by Scott and Calvert[20] that cystine is more effective than methionine in prevention of dystrophy.

Certain diets low in protein and especially the sulphur-containing amino acids, particularly cystine, have been found[21] to produce an acute massive hepatic necrosis in experimental animals. A vitamin E deficiency is reported to enhance the effect of such diets. Whereas added vitamin E exerts a preventive action upon the necrosis[21]. The enhanced permeability of cystine in the presence of α-tocopherol (Table 3) could be a plausible explanation for these observations on the causation and prevention of hepatic necrosis.

Thus the model studies reported in this paper indicate that the phenomenon of liquid membrane formation may also play a notable role in the causation and

prevention of various syndromes due to vitamin E
deficiency . It may be emphasized that since the
supporting membrane chosen in this study was a non-
specific, non-living membrane, the present study
highlights the role of passive transport in the
biological action.

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CHAPTER VII

SUMMARY

CHAPTER VII

SUMMARY

The Chapterwise summary of the work recorded in this thesis is given below:

Chapter I

Chapter 1 gives a brief accout of the Kesting's liquid membrane hypothesis and its biological implications. A discussion of the liquid membrane hypothesis of drug action vis-a-vis existing theories of drug action has been presented . At the end of the chapter, the tasks intended to be achieved in the thesis have also been brought out.

Chapter II

A general account of the experimental methods used in these investigations have been recorded in this Chapter.

Chapter III

The liquid membrane phenomenon in gonadal steroid hormones has been studied. Hydraulic permeability data have been obtained to demonstrate the

formation of liquid membranes by the gonadal steroid
hormones in association with sphingomyelin at the
supporting membrane/aqueous phase interface. Data on
the transport of several relevant neurotransmitters
viz adrenaline, noradrenaline, dopamine and serotonin
in the presence of the liquid membranes generated by
the sphingomyelin-gonadal steroid hormones mixture
have been obtained and discussed in the light of
the various physiological functions of the gonadal
steroid hormones. The data indicate that modification
in the permeability of the relevant neurotransmitters
in the presence of the liquid membranes is likely to
play a significant role in the physiological actions
of the gonadal steroid hormones.

Chapter IV

The liquid membrane phenomenon in prostaglandins
has been studied. Hydraulic permeability data have
been obtained to demonstrate the existence of the liquid
membrane in series with a supporting membrane generated
by the prostaglandins and also by the lecithin-cholesterol
prostaglandin mixtures. Data on the transport of the
relevant permeants in the presence of the liquid
membranes generated by the lecithin-cholesterol-

prostaglandin mixtures have been obtained and discussed in the light of the various biological actions of the prostaglandins. Studies have been conducted on prostaglandin E_1 and prostaglandin $F_{2\alpha}$.

Chapter V

Liquid membrane phenomena in 5-fluorouracil and its two derivatives: 1-(2-tetrahydrofuryl)-5-fluorouracil and 1-hexylcarbamoyl-5-fluorouracil have been studied. Modification in the transport of folic acid, vitamin B_{12} and a few relevant amino acids in the presence of the liquid membrane generated by these drugs in series with a supporting membrane has been studied. The data indicate that modification in the transport of the relevant permeants due to the liquid membranes likely to be generated by these drugs at their respective sites of action may also contribute to their anticancer activity.

Chapter VI

The liquid membrane phenomenon in vitamin E has been studied. Hydraulic permeability data have been obtained to demonstrate the existence of the liquid membranes in series with a supporting membrane generated

by α-tocopherol and also by the lecithin- cholesterol - α-tocopherol mixtures. Data on the transport of oestrogen, progesterone, cystine, methionine, creatinine and sodium, potassium and calcium ions in the presence of the liquid membrane generated by the lecithin-cholesterol-α-tocopherol mixture have been obtained and discussed in the light of the various syndromes caused by vitamin E deficiency. The data indicate that modification in the permeability of the various relevant permeants in the presence of liquid membranes is likely to play a significant role in causing and prevention of the various syndromes due to the deficiency of vitamin E.

List of Publications Based on the work
recorded in this thesis

1. Liquid membrane phenomenon in vitamin E: Studies on α-tocopherol Int. J. Pharm. ,$\underline{32}$, (1986)39-45.

2. Liquid membrane phenomena in prostaglandins: Studies on prostaglandin E_1 and prostaglandin $F_{2\alpha}$. J.Colloid Interface Sci., $\underline{117}$, (1987) 375-383.

3. Liquid membrane phenomena in anticancer drugs. Studies on 5-fluorouracil and its derivatives Int. J. Pharm. $\underline{38}$, (1987) 239-245.

4. Liquid membrane phenomenon in biological actions of gonadal steroid hormones. Indian J. Biochem. Biophys., (accepted for publication.)

REPRINTS

Liquid membrane phenomenon in vitamin E: studies on α-tocopherol

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Summary

The liquid membrane phenomenon in vitamin E has been studied. Hydraulic permeability data have been obtained to demonstrate the existence of the liquid membranes in series with a supporting membrane generated by α-tocopherol and also by the lecithin–cholesterol–α-tocopherol mixtures. Data on the transport of oestrogen, progesterone, cystine, methionine, creatinine and sodium, potassium and calcium ions in the presence of the liquid membrane generated by the lecithin–cholesterol–α-tocopherol mixture have been obtained and discussed in the light of the various syndromes caused by vitamin E deficiency. The data indicate that modification in the permeability of the various relevant permeants in the presence of liquid membranes is likely to play a significant role in causing and prevention of the various syndromes due to the deficiency of vitamin E.

Introduction

Recent studies (Bhise et al., 1982, 1983a and b, 1984a and b, 1985; Srivastava et al., 1984) on a variety of surface-active drugs belonging to different chemical and pharmacological categories, have revealed that modification in the transport of relevant permeants by the liquid membranes likely to be generated by them at the respective sites of action may be an important step common to the mechanism of action of all surface-active drugs.

α-Tocopherol is the most important tocopherol because it comprises about 90% of the tocopherols in animal tissues and exhibits maximum biological activity. It is distributed throughout the tissues of animals and man and its deficiency causes a variety of syndromes in the animal organism. Just by looking at the structure of α-tocopherol one suspects it to be surface-active in nature. Since according to Kesting's hypothesis (Kesting et al., 1968) surface-active agents, when added to aqueous phase, generate a surfactant layer liquid membrane at the interface, it is likely that the phenomenon of liquid membrane formation may play a role in the actions of α-tocopherol.

In the present study investigations were carried out to explore the role of liquid membrane phenomenon in the actions of α-tocopherol. Critical micelle concentration of α-tocopherol in water has been determined. The data on hydraulic permeability have been obtained to demonstrate: (1) the formation of a liquid membrane by α-tocopherol in series with the supporting membrane; and (ii) the incorporation of α-tocopherol in the lecithin–cholesterol liquid membrane existing in series with

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the supporting membrane. Transport of relevant permeants in the presence of the liquid membrane generated by the lecithin–cholesterol–α-tocopherol mixture has been studied and the data obtained have been discussed in the light of the various syndromes caused by vitamin E deficiency.

Materials and Methods

Lecithin (egg phosphatidyl choline) and progesterone (Patel Chest Institute, CSIR Centre for Biochemicals, Delhi), cholesterol (Centron Research Laboratories, Bombay), DL-α-tocopherol acetate (Sigma T3376), ethinyl oestradiol (Roussel Pharmaceuticals (India) Bombay), cystine, methionine and creatinine (all from Loba Chemie), sodium, potassium and calcium chlorides (all Analar grade) and distilled water distilled twice in an all-pyrex glass still were used in the present experiments.

Aqueous solutions of desired concentration of α-tocopherol, lecithin–cholesterol–α-tocopherol mixtures, ethinyl oestradiol and progesterone which are not so easily soluble in water were prepared by adding necessary volume of ethanolic stock solution of known concentration to aqueous phase with constant stirring. In the aqueous solutions thus prepared the final concentration of ethanol was never allowed to exceed 0.1% by volume because it was experimentally shown that a 0.1% solution of ethanol in water did not lower the surface tension of water to any measurable extent. The aqueous solutions of cystine, methionine, creatinine and chlorides of sodium, potassium and calcium were prepared in the usual way.

The critical micelle concentration (CMC) of aqueous α-tocopherol, aqueous lecithin and aqueous cholesterol were determined from the variation of their surface tension with concentration and were found to be 5.0×10^{-8} M, 1.599×10^{-5} M and 30.08×10^{-9} M, respectively. Surface tensions were measured using a Fisher tensiomat model 21.

The all-glass cell described earlier (Srivastava and Jakhar, 1981; Bhise et al., 1982) was used for the transport studies (Fig. 1). A Sartorius cellulose
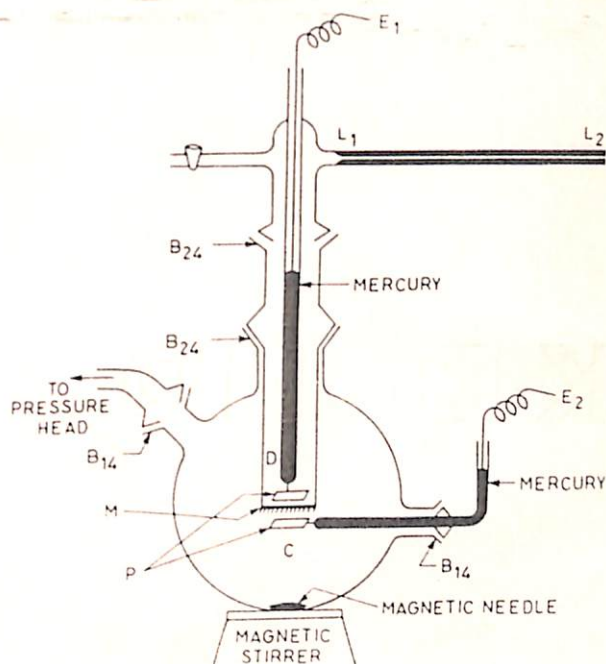

Fig. 1. Transport cell. M, supporting membrane; P, bright, platinum electrodes; E_1 and E_2, electrode terminals; L_1 and L_2, capillary.

acetate microfiltration membrane (Cat No. 11107, pore size 0.2 μm) of thickness 1×10^{-4} m and area 2.55×10^{-5} m^2 which in fact acted as a supporting membrane for the liquid membranes, divided the transport cell into two compartments C and D (Fig. 1).

To obtain the hydraulic permeability data, solutions of varying concentrations of α-tocopherol in water or in the aqueous solution of the lecithin–cholsterol mixtures of fixed composition were filled in compartment C of the transport cell (Fig. 1) and the compartment D was filled with water. The details of the method used for the hydraulic permeability measurements have been described earlier (Srivastava and Jakhar, 1981, 1982; Bhise et al., 1982, 1983b).

For solute permeability (σ) measurements for various permeants, namely oestrogen, progesterone, cystine, methionine, creatinine and sodium, potassium and calcium ions, the procedure described earlier (Bhise et al., 1982, 1983a and b, 1984a and b, 1985; Srivastava and Jakhar, 1982) was followed. Compartment C of the transport cell (Fig. 1) was filled with the solution of known

concentration of the permeant prepared in the aqueous solution of lecithin, cholesterol and α-tocopherol mixture of composition 1.919×10^{-5} M with respect to lecithin, 1.175×10^{-6} M with respect to cholesterol and 3.75×10^{-8} M with respect to α-tocopherol and compartment D was filled with distilled water. In control experiments, however, no α-tocopherol was used.

This particular composition, of the aqueous solution of lecithin–cholesterol–α-tocopherol mixture, used in the experiments for solute permeability measurements, which was derived from our earlier study (Srivastava and Jakhar, 1982) and the present data on hydraulic permeability, is the composition at which the lecithin liquid membrane compeletely covers the supporting membrane and is saturated with both cholesterol and α-tocopherol.

Since lecithin, cholesterol and α-tocopherol are all surface-active in nature and have both hydrophobic and hydrophilic parts in their structure, it is obvious that in the liquid membranes generated in these experiments the hydrophobic tails of these molecules will be preferentially oriented towards the hydrophobic supporting membrane and the hydrophilic moieties would be drawn outwards away from it.

The values of the solute permeability (ω) were estimated using the equation (Katchalsky and Kedem, 1962; Katchalsky and Curran, 1967):

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

where J_s is the solute flux, J_v is the volume flux per unit area of the membrane and $\Delta\pi$ is the osmotic pressure difference. The value of $\Delta\pi$ used in the calculation of ω from Eqn. 1 was the average of its value at the beginning of the experiment (t = 0) and at the end of the experiment.

All measurements were carried out at constant temperature using a thermostat set at $37 \pm 0.1°C$.

Estimations

The amounts of the various permeants transported to compartment D were estimated as follows:

(1) *Amino acids*. The amounts of cystine and methionine were estimated from the amount of their reaction products with ninhydrin measured at 570 nm (Moore and Stein, 1954) using a Bausch & Lomb Spectronic-20 spectrophotometer.

(2) *Creatinine*. The amount of creatinine was estimated using the method described in literature (Oser, 1965) in which creatinine was subjected to react with alkaline picrate to form an orange-coloured 'Jaffe complex', the intensity of which was measured spectrophotometrically at 520 nm.

(3) *Oestrogen*. The amount of ethinyl oestradiol was estimated by a chemical method (Brown, 1955) using quinol-H_2SO_4 as a colouring reagent and the colour developed was measured at 538 nm using a spectrophotometer.

(4) *Progesterone*. The amount of progesterone was estimated using a Cary 17-D Spectrophotometer at 242 nm the absorption maxima for progesterone (Heller, 1980).

(5) *Cations*. The amounts of sodium, potassium and calcium ions were determined using a flame-photometer (Model CL-22, Elico, India).

Results and Discussion

The hydraulic permeability data at various concentrations of α-tocopherol were, in all cases, found to be represented by the equation:

$$J_v = L_P \cdot \Delta P \tag{2}$$

where J_v represents the volume flux per unit area of the membrane, ΔP the applied pressure difference and L_P stands for the hydraulic conductivity coefficient. The values of L_P at various concentrations of α-tocopherol, estimated from the slopes of the J_v versus ΔP plots show a decreasing trend with the increase in concentration of α-tocopherol up to its CMC beyond which they become more or less constant (Table 1). This trend indicates progressive coverage of the supporting membrane with the liquid membrane generated by α-tocopherol in accordance with the Kesting's hypothesis (1968). At the CMC the α-tocopherol liquid membrane completely covers the supporting membrane. Analysis of the transport data (Table 1) in light of mosaic membrane model

TABLE 1

VALUES OF L_p AT VARIOUS CONCENTRATIONS OF α-TOCOPHEROL

Concentration $\times 10^8$ M	0.00	1.25 (0.25 CMC)	2.50 (0.5 CMC)	3.75 (0.75 CMC)	5.00 (1 CMC)	10.00	15.00
$L_p^a \times 10^8$ (m³·s⁻¹·N⁻¹)	4.891 ± 0.137	4.473 ± 0.189	4.078 ± 0.121	3.669 ± 0.113	3.170 ± 0.077	3.112 ± 0.141	3.098 ± 0.042
$L_p^b \times 10^8$ (m³·s⁻¹·N⁻¹)	–	4.461 ± 0.122	4.031 ± 0.107	3.600 ± 0.092	–	–	–

^{a} Experimental values.
^{b} Calculated values using mosaic model.

(Spiegler and Kedem, 1966; Sherwood et al., 1967; Harris et al., 1976) furnishes further support in favour of the formation of the liquid membrane in series with the supporting membrane. Following the argument given in earlier publications (Srivastava and Jakhar, 1981, 1982; Bhise et al., 1982, 1983a and b, 1984a and b, 1985) it follows that if concentration of the surfactant is n times its CMC, n ≤ 1, the value of L_p should be equal to $[(1 - n)L_p^c + n L_p^s]$ where the superscripts c and s, respectively, represent the bare supporting membrane and the supporting membrane completely covered with the surfactant layer liquid membrane. Functionally, in the present case, the values of L_p^c and L_p^s would respectively represent the values of L_p for O and the CMC of α-tocopherol. The values of L_p thus computed at various concentrations of α-tocopherol below its CMC match with the experimentally determined values (Table 1).

Information about the incorporation of α-tocopherol in the liquid membranes generated at the interface by lecithin–cholesterol mixture can be gathered from the hydraulic permeability data for solutions of various concentrations of α-tocopherol prepared in the aqueous solutions of lecithin–cholesterol mixtures of fixed composi-

tion, i.e. 1.919×10^{-5} M with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol, which in fact is the composition at which the liquid membrane generated by lecithin completely covers the interface and is saturated with cholesterol (Srivastava and Jakhar, 1982). The values of L_p at various concentrations of α-tocopherol estimated, from the J_v vs ΔP plots, which in this case also were found to be in accordance with Eqn. 2 are recorded in Table 2. The decreasing trend in the values of L_p which continues up to the α-tocopherol concentration equal to 3.75×10^{-8} M (Table 2) indicates that more and more of α-tocopherol is incorporated in the lecithin–cholesterol liquid membrane generated at the interface and at concentration equal to 3.75×10^{-8} M the lecithin–cholesterol liquid membrane is saturated with α-tocopherol. In order to ascertain whether the added α-tocopherol reaches straight up to the interface or not, surface tensions of solutions of various concentrations of α-tocopherol prepared in the aqueous solution of the lecithin–cholesterol mixture of composition 1.919×10^{-5} M with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol, were measured. The surface tension of the aqueous solution of the lecithin–cholesterol mixture showed a further de-

TABLE 2

VALUES OF L_p AT VARIOUS CONCENTRATIONS OF α-TOCOPHEROL IN LECITHIN–CHOLESTEROL–α-TOCOPHEROL MIXTURES ^{a}

Concentration $\times 10^8$ M	0.00	1.25	2.50	3.75	5.00	10.00
$L_p \times 10^8$ (m³·s⁻¹·N⁻¹)	1.575 ± 0.084	1.402 ± 0.045	1.296 ± 0.012	1.178 ± 0.011	1.185 ± 0.031	1.164 ± 0.033

^{a} Lecithin and cholesterol concentrations kept constant at 1.919×10^{-5} M and 1.175×10^{-6} M. respectively.

crease on addition of α-tocopherol and the decreasing trend continued up to the α-tocopherol concentration equal to 3.75×10^{-8} M. This trend indicates that the added α-tocopherol reaches deep up to the interface in the liquid membrane generated by the lecithin-cholesterol mixture in series with the supporting membrane.

Solute permeability data

Data on the solute permeability (σ) of several permeants, namely oestrogen, progesterone, cystine, methionine, creatinine and cations (Na⁺, K⁺ and Ca²⁺ ions), in the presence of the liquid membranes generated by the mixture of lecithin, cholesterol and α-tocopherol in series with the supporting membrane are recorded in Table 3. The data appear to be relevant to causation of various syndromes in animal organisms due to deficiency of vitamin E, i.e. α-tocopherol.

TABLE 3

SOLUTE PERMEABILITY (σ) OF VARIOUS PERMEANTS IN PRESENCE OF LECITHIN–CHOLESTEROL–α-TOCOPHEROL MIXTURE [a]

	$\sigma^{b} \times 10^{9}$ (mol·s⁻¹·N⁻¹)	$\sigma^{c} \times 10^{9}$ (mol·s⁻¹·N⁻¹)
Methionine [d]	8.79 ± 0.27	6.25 ± 0.24
Cystine [e]	2.62 ± 0.09	4.66 ± 0.56
Creatinine [f]	0.27 ± 0.03	0.29 ± 0.02
Ethinyl oestradiol [g]	5.50 ± 0.40	4.14 ± 0.24
Progesterone [h]	4.90 ± 0.38	4.11 ± 0.20
Sodium (chloride) [i]	0.12 ± 0.01	0.12 ± 0.01
Potassium (chloride) [j]	0.13 ± 0.01	0.14 ± 0.01
Calcium (chloride) [k]	0.16 ± 0.03	0.18 ± 0.01

[a] Lecithin concentration, 1.919×10^{-5} M; cholesterol concentration, 1.175×10^{-6} M; α-tocopherol concentration, 3.75×10^{-8} M.
[b] Control value when no α-tocopherol was used.
[c] Lecithin–cholesterol–α-tocopherol mixture in compartment C together with the permeant.
[d] Initial concentration 100 mg/l.
[e] Initial concentration 100 mg/l.
[f] Initial concentration 1 g/l.
[g] Initial concentration 50 mg/l.
[h] Initial concentration 100 mg/l.
[i] Initial concentration 5.382 g/l.
[j] Initial concentration 10.430 g/l.
[k] Initial concentration 0.222 g/l.

Except for the work cited by Wagner and Folkers (1963) there is enough evidence to indicate that vitamin E is essential for normal reproduction in several mammalian species (Marks, 1962; Mandel and Cohn, 1980) and its deficiency is known to cause habitual abortions. The fundamental mechanism by which vitamin E deficiency interferes with reproduction is obscure (Mandel and Cohn, 1980). The present data (Table 3) on the impedimenting in the transport of oestrogen and progesterone in the presence or α-tocopherol may offer an explanation for occurrence of habitual abortions caused by vitamin E deficiency.

It is not only the high concentrations of oestrogen and progesterone but also a proper ratio of their concentrations which is essential for the maintenance of pregnancy (Kincl, 1971). As the present data indicate, the deficiency of vitamin E in the membranes of the uterus would enhance the outflow of oestrogen and progesterone to an unequal extent. This outflow would disturb the oestrogen–progesterone ratio resulting in the failure of pregnancy.

In many species, deficiency of vitamin E leads to the development of muscular dystrophy. Metabolic disturbances during muscular dystrophy include increased water content of the tissues, changes in electrolyte pattern and increased excretion of creatine in urine–creatinurea (Mason, 1944). The values of solute permeability, σ, for the cations and also for creatinine in the presence of α-tocopherol do not show any significant difference in comparison to the values obtained from the control experiment where no α-tocopherol was used. The data on hydraulic permeability (Table 2), however, appear relevant to causation of increased water content of the tissues and creatinurea. The data in Table 2 imply that the cell membranes deficient in vitamin E are likely to be more permeable to water which may be one of the factors responsible for the increased water content of the tissue. It has been suggested (Beard, 1941; Mason, 1944) that creatinurea in nutritional muscular dystrophy might be due to hydration of creatinine to creatine due to increased water content of the tissues–creatinine is formed inside the cells as a result of creatine metabolism. The alteration in the normal water balance of tissues is a

consistent finding in the biochemical and histological examinations of tissues affected by vitamin E depletion (Mason, 1944). Nitowsky et al. (1962) have shown that tocopherol can decrease the elevated creatine excretions of children with cystic fibrosis.

Dam and associates (1952) have shown that muscular dystrophy in chicks could be prevented by supplementing the diet with either vitamin E or cystine. Later Machlin and Shalkop (1956) showed that cystine and methionine were equally effective in prevention of dystropy. However, Scott (1962) and Scott and Calvert (1962) have reported that cystine is more effective than methionine. The present data (Table 3) show that the permeability of cystine is enhanced and that the amount of methionine was reduced in the presence of α-tocopherol. This observation is consistent with the inference drawn by Scott (1962) and Scott and Calvert (1962) that cystine is more effective than methionine in prevention of dystrophy.

Certain diets low in protein and especially in the sulfur-containing amino acids, particularly cystine, have been found (Harper, 1975) to produce an acute massive hepatic necrosis in experimental animals. A vitamin E deficiency is reported to enhance the effects of such diets, whereas added vitamin E exerts a preventive action upon the necrosis (Harper, 1975). The enhanced permeability of cystine in the presence of α-tocopherol (Table 3) could be a plausible explanation for these observations on the causation and prevention of hepatic necrosis.

Thus the model studies reported in this paper indicate that the phenomenon of liquid membrane formation may also play a notable role in the causation and prevention of various syndromes due to vitamin E deficiency. It may be emphasized that since the supporting membrane chosen in this study was a non-specific, non-living membrane, the present study highlights the role of passive transport in biological action.

Acknowledgements

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Liquid Membrane Phenomena in Prostaglandins: Studies on Prostaglandin E$_1$ and Prostaglandin F$_{2\alpha}$

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The liquid membrane phenomenon in prostaglandins has been studied. Hydraulic permeability data have been obtained to demonstrate the existence of the liquid membrane in series with a supporting membrane generated by the prostaglandins and also by the lecithin–cholesterol prostaglandin mixtures. Data on the transport of the relevant permeants in the presence of the liquid membranes generated by the lecithin–cholesterol–prostaglandin mixtures have been obtained and discussed in the light of the various biological actions of the prostaglandins. Studies have been conducted on prostaglandin E$_1$ and prostaglandin F$_{2\alpha}$. © 1987 Academic Press, Inc.

INTRODUCTION

Recent studies (1–13) on a wide variety of structurally dissimilar surface-active drugs belonging to different pharmacological categories have revealed that modification in the transport of relevant permeants by the liquid membranes they generate at the respective action sites may be an important step common to the mechanism of action of all surface-active drugs. Transport of relevant permeants through the liquid membranes generated by the drugs, though passive in nature, has been shown to contribute significantly to their action. A detailed discussion of this point of view has been presented in a recent review (14). In the present communication investigations exploring the role of the phenomenon of liquid membrane formation in the actions of prostaglandins are reported. The investigations have been conducted on prostaglandin E$_1$ (PGE$_1$) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$).

The prostaglandins are among the most prevalent autacoids and have been detected in almost every tissue and body fluid; they produce, in minute amounts, a remarkably broad spectrum of effects that embrace practically every biological function. No other autacoids show more numerous and diverse effects than do prostaglandins.

Just by looking at the structures of prostaglandins their surface-active nature becomes apparent prima facie. One can therefore suspect that prostaglandins, when added to an aqueous phase, according to Kesting's liquid membrane hypothesis (15), would generate surfactant layer liquid membranes at the interface.

In the present studies the data on hydraulic permeability in the presence of various concentrations of the prostaglandins have been obtained to demonstrate the formation of the surfactant layer liquid membrane they generate in series with a supporting membrane. The data on the hydraulic permeability in the presence of varying concentrations of the prostaglandins in a mixture of lecithin and cholesterol of fixed composition have been utilized to demonstrate incorporation of the prostaglandins into the liquid membrane generated by the lecithin–cholesterol mixture. Transport of several relevant permeants through the liquid membranes, generated by the lecithin–cholesterol–prostaglandin mixtures, in series with a supporting membrane has been studied and the data obtained have

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been discussed in light of the reported biological effects of the prostaglandins. In these model studies a Sartorius cellulose nitrate microfiltration membrane was deliberately chosen as supporting membrane to highlight the role of passive transport through the liquid membrane in the pharmacological actions of prostaglandins.

EXPERIMENTAL

Materials

Lecithin (phosphatidylcholine from egg yolk) and progesterone (Patel Chest Institute, CSIR Centre for Biochemicals, Delhi), cholesterol (Centron Research Laboratories, Bombay), prostaglandin E_1 (Sigma p-5515) and histamine (Sigma H-7250), prostaglandin $F_{2\alpha}$ (33735, Serva, Germany), ethinyl estradiol (Roussel Pharmaceuticals (India) Ltd., Bombay), glucose and γ-aminobutyric acid (BDH, England), glycine and adrenaline hydrogen tartarate (Loba Chemie), sodium and potassium chlorides (BDH AR Grade), and water twice distilled in an all-Pyrex glass still were used in the present experiments.

The aqueous solutions of lecithin–cholesterol mixtures of the desired composition were prepared by adding the necessary volume of ethanolic stock solution of known concentration of the respective components to the aqueous phase with constant stirring. The aqueous solutions with the desired concentrations of ethinyl estradiol and progesterone, which are not easily soluble in water, were also prepared by adding the necessary volume of ethanolic stock solution of known concentration to the aqueous phase. In all aqueous solutions thus prepared the final concentration of ethanol was never allowed to exceed 0.1% by volume. The values of critical micelle concentrations (CMCs) of PGE_1, $PGF_{2\alpha}$, lecithin, and cholesterol as determined from the variation of surface tensions with concentrations were found to be 1×10^{-8} M, 9.3×10^{-8} M, 12.952 ppm, and 30.08×10^{-9} M, respectively. The CMC values of lecithin and cholesterol were in agreement with the values determined

earlier (16, 17). The surface tensions were measured using a Fisher Tensiomat Model 21.

Methods

The all-glass cell described earlier (16, 18) was used for the transport studies. It essentially consisted of two compartments C and D separated by a Sartorius cellulose nitrate microfiltration membrane (Catalog No. 11307) 1×10^{-4} m thick and 2.55×10^{-5} m^2 in area which acted as a supporting membrane for the liquid membranes.

To obtain the hydraulic permeability data which were utilized to demonstrate the formation of liquid membranes by prostaglandins, compartment C of the transport cell (Fig. 1, Ref. (16) or (18)) was filled with aqueous solutions of varying concentrations of prostaglandins, PGE_1 or $PGF_{2\alpha}$, and compartment D was filled with distilled water. Known pressures were applied to compartment C and the consequent movement of the liquid meniscus in the capillary L_1L_2 of known diameter (Fig. 1, Ref. (16) or (18)) was measured using a cathetometer reading up to 0.001 cm and a stopwatch reading up to 0.1 s. During the hydraulic permeability measurements the electrodes E_1 and E_2 (Fig. 1, Ref. (16), (18)) were short-circuited so that the electroosmotic backflow due to the streaming potentials developed across the membrane did not interfere with the hydraulic permeability measurements. The details of the procedure are described in earlier publications (16–18).

Similarly, to obtain the hydraulic permeability data which were utilized to demonstrate the incorporation of prostaglandins into the liquid membranes generated by lecithin–cholesterol mixtures, compartment C of the transport cell was filled with solutions of varying concentrations of prostaglandins prepared in the aqueous solutions of the mixture of lecithin and cholesterol of fixed composition (15.542 ppm with respect to lecithin and 1.175 $\times 10^{-6}$ M with respect to cholesterol) and compartment D (Fig. 1 of Ref. (16) or (18)) was filled with distilled water. This particular

composition, i.e., 15.542 ppm with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol, of the lecithin–cholesterol mixture was chosen for preparing the solutions of varying concentrations of prostaglandins because it has been shown in earlier studies (17) that at this composition the liquid membrane generated by lecithin completely covers the interface and is fully saturated with cholesterol.

For solute permeability (ω) measurements for the relevant permeants the method outlined in earlier publications (1–13, 17, 18) was used. Compartment C of the transport cell (Fig. 1 of Refs. (16, 18)) was filled with the solution of known concentration of the permeant prepared in the aqueous solution of lecithin, cholesterol, and one of the prostaglandins (PGE$_1$ and PGF$_{2\alpha}$) under study and compartment D was filled with distilled water. The composition of the aqueous solution of the lecithin–cholesterol–prostaglandin mixture used in the solute permeability experiments was such that the liquid membrane generated by lecithin, in series with the supporting membrane, was completely saturated with both cholesterol and the prostaglandin under study. This composition was derived from our earlier study (17) and from present data on hydraulic permeability in the presence of varying concentrations of prostaglandins in the lecithin–cholesterol mixture of fixed composition. Since lecithin, cholesterol, and prostaglandins are all surface active in nature, i.e., they have both hydrophilic and hydrophobic parts in their structure, it is obvious that in the liquid membranes generated in the solute permeability experiments, the hydrophobic tail of these molecules will be oriented preferentially toward the hydrophobic supporting membrane and the hydrophilic moieties will be drawn away from it.

The condition of no net volume flux ($J_v = 0$) was imposed on the system by adjusting the pressure head attached to compartment C of the transport cell (Fig. 1, Ref. (16, 18)) in such a way that the liquid meniscus in the capillary L_1L_2 remained stationary. After a known period of time which was of the order of several hours, the amount of permeant transported to compartment D was estimated. The values of solute permeability ω were estimated using the equation (19)

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

where J_s is the solute flux, the solute transported per unit time per unit area of the membrane, and $\Delta \pi$ is the osmotic pressure difference. The value of $\Delta \pi$ in the calculation of ω using Eq. [1] was the average of its values at the beginning of the experiment ($t = 0$) and at the end of the experiment.

All measurements were made at constant temperature using a thermostat set at 37 $\pm$ 0.1°C.

The amounts of the various permeants transported to compartment D were estimated as follows:

(i) *Glucose.* The amount of glucose was estimated using dinitrosalicylic acid. The reaction is followed by measuring the extinction at 540 nm (20).

(ii) *Histamine.* The amount of histamine was estimated by a method involving condensation of histamine with *o*-phthaldehyde to yield a highly fluorescent product (21).

(iii) *Adrenaline.* The amount of adrenaline (hydrogen tartrate) was estimated by UV absorption at 281 nm in 0.1 N hydrochloric acid (22).

(iv) *Ethinyl estradiol.* The amount of ethinyl estradiol was estimated by a chemical method (23) using quinol-H$_2$SO$_4$ as a coloring reagent and the color developed was measured at 538 nm using a spectrophotometer.

(v) *Progesterone.* The amount of progesterone was estimated using a Cary 17-D spectrophotometer at 242 nm, the absorption maxima for progesterone (24).

(vi) *Amino acids.* The amounts of glycine and γ-amino butyric acid (GABA) were estimated from the amount of their reaction products with ninhydrin measured at 570 nm (25).

(vii) *Cations.* The amounts of sodium and potassium ions were determined using a flame photometer (Model CL-22, Elico).

RESULTS AND DISCUSSION

The hydraulic permeability data at various concentrations of prostaglandins—both PGE$_1$ and PGF$_{2\alpha}$—in all the cases presently studied were found to be represented by the proportional relationship

$$J_v = L_p \Delta P, \qquad [2]$$

where J_v represents the volume flux per unit area of the membrane, ΔP is the applied pressure difference, and L_p stands for the hydraulic conductivity coefficient. The values of L_p at various concentrations of the two prostaglandins estimated from the slopes of the J_v versus ΔP plots are recorded in Tables I and II. The values of L_p progressively decrease with an increase in the concentrations of the prostaglandins up to their CMCs, beyond which they become more or less constant. This trend in the values of L_p is in accordance with Kesting's liquid membrane hypothesis and is indicative of the formation of liquid membranes by the prostaglandins in series with the supporting membrane. According to Kesting's hypothesis (15), which was originally propounded to account for enhanced salt rejection in reverse osmosis due to the addition of surfactant additives to saline feed, when a surfactant is added to an aqueous phase the surfactant layer which forms spontaneously at the interface acts as a liquid membrane and modifies mass transfer across the interface. 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. The values of L_p were further analyzed in the light of the mosaic model (26–28) to obtain additional evidence in favor of the formation of liquid membranes in series with the supporting membrane. It has been shown in earlier publications (e.g., (16, 17)) that if the concentration of the surfactant is n times its CMC, $n \leq 1$, the value of L_p

TABLE I

Values of L_p at Various Concentrations of Prostaglandin E$_1$ and Prostaglandin F$_{2\alpha}$

	Concentration ($\times 10^4$ M)									
	0.0	0.2 (0.2 CMC)	0.4 (0.4 CMC)	0.6 (0.6 CMC)	0.8 (0.8 CMC)	1.0 (1 CMC)	1.6	2.0
Prostaglandin E$_1$								
$L_p^a \times 10^8$ (m^3 s^{-1} N^{-1})	5.971 ±0.085	5.616 ±0.065	5.082 ±0.122	4.746 ±0.054	4.355 ±0.050	3.851 ±0.049	3.794 ±0.028	3.771 ±0.095
$L_p^b \times 10^8$ (m^3 s^{-1} N^{-1})	—	5.547 ±0.078	5.123 ±0.070	4.699 ±0.063	4.275 ±0.056	—	—	—
	0.0	2.32 (0.25 CMC)	4.65 (0.5 CMC)	6.97 (0.75 CMC)	9.30 (1 CMC)	18.60	27.90	
Prostaglandin F$_{2\alpha}$								
$L_p^a \times 10^8$ (m^3 s^{-1} N^{-1})	5.971 ±0.085	4.958 ±0.141	4.016 ±0.125	3.067 ±0.086	1.997 ±0.137	1.930 ±0.051	1.907 ±0.105	
$L_p^b \times 10^8$ (m^3 s^{-1} N^{-1})	—	4.977 ±0.131	3.984 ±0.134	3.013 ±0.115	—	—	—	

^{a} Experimental values.
^{b} Calculated values using mosaic model.

TABLE II

Values of L_p at Various Concentrations of Prostaglandin E_1 and Prostaglandin $F_{2\alpha}$ in Lecithin–Cholesterol Mixtures[a]

	Concentration ($\times 10^8$ M)					
	0.0	0.2	0.4	0.6	0.8	1.0
Prostaglandin E_1 $L_p \times 10^8$ (m³ s⁻¹ N⁻¹)	5.550 ± 0.136	5.158 ± 0.169	4.824 ± 0.116	4.529 ± 0.106	4.640 ± 0.092	4.600 ± 0.055
	0.0	2.32	4.65	6.97	9.30	18.60
Prostaglandin $F_{2\alpha}$ $L_p \times 10^8$ (m³ s⁻¹ N⁻¹)	5.550 ± 0.136	4.995 ± 0.028	4.555 ± 0.035	4.087 ± 0.025	4.055 ± 0.025	4.055 ± 0.058

[a] Lecithin and cholesterol concentrations kept constant at 15.542 ppm and 1.175×10^{-6} M, respectively.

should be equal to $[(1 - n)L_p^c + nL_p^s]$, where the superscripts c and s represent the values for the bare supporting membrane and the supporting membrane completely covered with the surfactant layer liquid membrane, respectively. Functionally, L_p^c and L_p^s represent the values when the surfactant concentration equals 0 and CMC, respectively. The values of L_p, thus computed at several concentrations of the two prostaglandins below their CMCs match the corresponding experimental values (Table I) lending further evidence in favor of the formation of liquid membranes by the prostaglandins in series with the supporting membrane.

Information on the incorporation of prostaglandins into the liquid membrane generated by the lecithin-cholesterol mixture can be obtained from the hydraulic permeability data (Table II) at varying concentrations of the prostaglandins in the lecithin-cholesterol mixture of fixed composition—15.542 ppm with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol. The data (Table II) reveal that as the concentration of the prostaglandins is increased, holding the concentration of lecithin and cholesterol constant, the value of L_p, which measures the reciprocal of the resistance to volume flow, decreases. The decreasing trend in the values of L_p continues up to a PGE_1 concentration equal to 0.6 $\times 10^{-8}$ M and a $PGF_{2\alpha}$ concentration equal to 6.97×10^{-8} M, and thereafter the values of L_p become more or less constant. This trend in the values of L_p (Table II) is indicative of the strengthening of the hydrophobic core of the liquid membrane generated by the lecithin-cholesterol mixture at the interface due to its incorporation of the prostaglandins. It is also apparent from the data (Table II) that at a concentration equal to 0.6×10^{-8} M the lecithin-cholesterol liquid membrane is saturated with PGE_1, and at concentration equal to 6.97×10^{-8} M the lecithin-cholesterol liquid membrane is saturated with $PGF_{2\alpha}$. In order to ascertain whether the added prostaglandin reaches straight to the interface or not, surface tensions of solutions of various con-

centrations of the prostaglandins—both PGE₁ and PGF$_{2a}$,—prepared in the aqueous solutions of lecithin–cholesterol mixtures of composition 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 the lecithin cholesterol mixture showed a further decrease upon addition of the prostaglandins. The decreasing trend in the values of the surface tensions continued to a 0.6×10^{-8} M concentration in the case of PGE₁ and to a 6.97×10^{-8} M concentration in the case of PGF$_{2a}$. This trend indicates that the added prostaglandins, both PGE₁ and PGF$_{2a}$, reach deep into the interface of the liquid membranes generated by the lecithin–cholesterol mixtures in series with the supporting membrane.

Solute Permeability Data

The data, recorded in Table III on the solute permeability (ω) of various permeants in the presence of the liquid membranes generated by PGE₁ and PGF$_{2a}$ in association with the lecithin cholesterol mixtures, appear relevant to the various reported pharmacological actions of the prostaglandins. *The values in Table III are expressed as arithmetic mean ± standard deviation*—based on the 15 repeats for each value of ω.

The present data (Table III) show that the solute permeability (ω) for glucose is increased in the presence of both PGE₁ and PGF$_{2a}$, the increase in the presence of PGE₁ being much larger than the increase in the presence of PGF$_{2a}$. This observation on the increase in permeability of glucose is consistent with the literature reports, particularly in the case of PGE₁. It is documented (29, 30) that in isolated adipose tissues PGE₁ stimulates glucose uptake.

Cardiac output is generally increased by the prostaglandins of E and F series (31). It is also known (32) that adrenaline is a powerful cardiac stimulant and enhances cardiac output by acting on β_1 receptors. The data obtained in the present study (Table III) indicate that transport of adrenaline is increased in the presence of the liquid membranes generated by the prostaglandins. This observation suggests that increased permeability of adrenaline due to the prostaglandins present in the membranes of myocardial cells facilitating inter-

TABLE III

Solute Permeability (ω)^a of Various Permeants in Presence of Liquid Membranes Generated by Prostaglandin E₁ (ω_1) and Prostaglandin F$_{2a}$ (ω_2) in Lecithin–Cholesterol Mixtures^b along with the Control Values (ω_0) When no Prostaglandin Was Used

Permeants	Initial Concentration (mg liter^{-1})	$\omega_0 \times 10^9$ (mole s^{-1} N^{-1})	$\omega_1{}^c \times 10^9$ (mole s^{-1} N^{-1})	$\omega_2{}^d \times 10^9$ (mole s^{-1} N^{-1})
Glucose	10	0.288 ± 0.030	0.412 ± 0.070	0.360 ± 0.011
Histamine	10	0.392 ± 0.019	0.244 ± 0.007	0.229 ± 0.001
Adrenaline	100	1.592 ± 0.038	1.697 ± 0.026	2.216 ± 0.009
Ethinyl estradiol	50	2.280 ± 0.046	3.135 ± 0.035	3.424 ± 0.068
Progesterone	100	0.198 ± 0.032	0.697 ± 0.093	0.279 ± 0.006
Glycine	100	1.517 ± 0.061	2.284 ± 0.059	1.279 ± 0.034
γ-Amino butyric acid	200	0.916 ± 0.012	1.170 ± 0.024	1.153 ± 0.039
Sodium chloride	5.382	0.133 ± 0.008	0.196 ± 0.012	0.197 ± 0.002
Potassium chloride	10.430	0.122 ± 0.008	0.055 ± 0.001	0.101 ± 0.003

^{a} Values of ω are reported as arithmetic means of 15 repeats $\pm$ SD.
^{b} Lecithin concentration 15.542 ppm; cholesterol concentration, 1.175×10^{-6} M.
^{c} Prostaglandin E₁ concentration, 0.65×10^{-8} M.
^{d} Prostaglandin F$_{2a}$ concentration, 8.5×10^{-8} M.

action with β_1 receptors may also be a contributing factor to the reported increase in cardiac output by the prostaglandins.

Prostaglandins of E series are known to inhibit the gastric acid secretion stimulated by feeding histamine (33, 34) and this has raised the possibility of the therapeutic utility of certain methylated analogs of prostaglandins for peptic ulcers (35). The gastric acid secretion by histamine is exerted through H_2 receptors and is blocked by H_2 receptor antagonists (36). It has been indicated recently (9) that an impediment in the transport of histamine due to the liquid membranes which are likely to be generated by the H_2 receptor antagonists, drugs like cimetidine and ranitidine, at the site of action may also contribute to their H_2-antagonistic action. The present data on the transport of histamine in the presence of the PGE_1 liquid membrane (Table III) appear relevant to the reported (33, 34) inhibition of gastric acid secretion by the PGE_1. It appears that the resistance offered by the PGE_1 to the transport of histamine, impeding its access to the H_2 receptors, may also be a cause of the inhibition of histamine-induced gastric acid secretion from the parietal cells. Although histamine transport is also impeded in the presence of $PGF_{2\alpha}$ (Table III), the relevance of this observation in the context of gastric acid secretion is not clear.

Table III reveals that in the case of PGE_1 the transport of both glycine and GABA is enhanced whereas in the case of $PGF_{2\alpha}$ the transport of GABA is enhanced and that of glycine is impeded. The enhancement in the transport of glycine and/or GABA leading to their increased concentration in the brain could also be the reason for reported (37–39) effects such as sedation, stupor, catatonia, etc., induced by the administration of prostaglandins, particularly PGE_1, in animals.

It is reported (37) that in the intact central nervous system of a chloralose-anesthetized chick, intravenous administration of $PGF_{2\alpha}$ potentiates the crossed extensor reflex while PGE_1 inhibits it. The opposing trends observed in the transport of glycine (Table III), which

is known (40) to be utilized by the inhibitory interneurons of the spinal cord, may be relevant to the reported potentiation and inhibition of the crossed extensor reflex in chicks.

Prostaglandins have been used as abortive agents (41). The present data on the permeability of estrogen and progesterone (Table III) appear relevant to their abortive actions. Not only high concentrations of estrogen and progesterone but also a proper ratio of their concentrations are essential for the maintenance of pregnancy (42). As the present data indicate, the presence of high concentrations of prostaglandins in the membranes of the uterus would enhance the outflow of estrogen and progesterone to an unequal extent. This outflow would not only decrease the concentrations of estrogen and progesterone but also disturb the estrogen–progesterone ratio resulting in the failure of pregnancy.

The present data on the permeability of estrogen and progesterone also appear relevant to the causation of primary dysmenorrhea. There is substantial evidence to indicate that prostaglandin is a major causal factor in primary dysmenorrhea (43). Drugs having prostaglandin synthetase inhibitory activity have been reported to be effective in the treatment of dysmenorrhea. The effectiveness of oral contraceptives in the treatment of dysmenorrhea is also well established (43). These observations appear to indicate that the enhanced permeability (outflow) of estrogen and progesterone in the presence of the increased concentration of prostaglandins, particularly $PGF_{2\alpha}$, in the endometrium may also be a factor responsible for dysmenorrhea.

Prostaglandins of E and F series are present in the renal medulla. Renal prostaglandins have been implicated in antihypertensive action (44). It is suggested that prostaglandins may exert an antihypertensive action, acting either as peripheral vasodilators or by promoting diuresis with sodium loss, i.e., natriuresis (44). The enhanced permeability to sodium ions in the presence of prostaglandins as observed in the present experiment appears consistent with the latter mechanism. Sodium

reabsorption in proximal tubule is active in nature and is mediated by carbonic anhydrase (45). In addition to forces moving sodium ion and water out of the proximal tubule there is a component of leakage back across the tubular epithelium into the lumen of the proximal nephron (46). The back leak is passive in nature and its amount is influenced by peritubular osmotic pressure (46). The increased passive transport of sodium ions in the presence of prostaglandins (Table III) may thus offer an explanation for the diuretic and natriuretic effects of the prostaglandins due to the back-leak mechanism leading to their antihypertensive action.

The toxin *Vibrio cholerae* affects electrolyte handling by the epithelial cells of the intestinal mucosa in such a way that there is hypersecretion into the gut resulting in the profuse watery stools that characterize cholera. It has been suggested (47) that the toxin acts by stimulating prostaglandin synthesis. The enhanced permeability of sodium ions in the presence of the prostaglandins, as observed in the present study (Table III), suggests that a back-leak mechanism similar to the one proposed in the case of the natriuretic and diuretic effects of the prostaglandins (46) may also explain the hypersecretion into the lumen of the intestines due to the increased concentration of the prostaglandin in the epithelial cells of the intestinal mucosa.

$PGF_{2\alpha}$ does not affect the transport of potassium ions significantly (Table III). In the presence of PGE_1, however, a decrease in the transport of potassium ions is observed (Table III). The observation on the decreased permeability of potassium ions may be relevant to the causation of Bartter's syndrome. Bartter's syndrome, an unusual and complex disorder which is characterized by, among other symptoms, hypokalemia, i.e., excessive loss of potassium, has been associated with excessive production of renal prostaglandins (48). This is obvious from the fact that Bartter's syndrome has been successfully treated with drugs like indomethacin and aspirin (49–52) which have prostaglandin synthetase inhibitory ac-

tivity. Although potassium reabsorption in proximal tubules is active in nature, the present data suggest that impediments in the transport of potassium ion due to the increased concentration of the prostaglandins in the tubular cells may also contribute to the urinary potassium wasting leading to hypokalemia.

The present study thus indicates that the phenomenon of liquid membrane formation may play a significant role in the biological action of the prostaglandins.

ACKNOWLEDGMENT

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Liquid membrane phenomena in anticancer drugs. Studies on 5-fluorouracil and its derivatives

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Summary

Liquid membrane phenomena in 5-fluorouracil and its two derivatives: 1-(2-tetrahydrofuryl)-5-fluorouracil and 1-hexyl-carbamoyl-5-fluorouracil have been studied. Modification in the transport of folic acid, vitamin B_{12} and a few relevant amino acids in the presence of the liquid membrane generated by these drugs in series with a supporting membrane has been studied. The data indicate that modification in the transport of the relevant permeants due to the liquid membranes likely to be generated by these drugs at their respective sites of action may also contribute to their anticancer activity.

Introduction

Recent studies (Bhise et al., 1984a and b, 1985a and b; Srivastava et al., 1984, 1985, 1986; Tandon et al., 1986) on a wide variety of structurally different surface-active drugs belonging to different pharmacological categories have revealed that liquid membranes likely to be generated at the site of action of the respective drugs, acting as a barrier modifying the transport of relevant permeants to these sites, might be an important step common to the mechanism of action of all surface-active drugs. It has been shown in the same studies that the modification in the transport of the relevant permeants to the site of action, due to the liquid membrane generated by the drugs,

makes a significant contribution to their biological action. A detailed discussion of this point of view and its implications has been presented by Srivastava, et al. (1984). One of the important implications is that in a series of structurally related drugs which are congeners of a common chemical moiety and which act by altering the permeability of cell membranes, any structural variation which increases the hydrophobicity of the compound will increase the potency of the drug while any alteration of the hydrophilic moieties of the drug may change the nature of its action qualitatively (Srivastava et al., 1984).

It has been shown by Iigo et al. (1978) that the newly synthesized (Ozaki et al. 1977) 1-hexylcarbamoyl-5-fluorouracil (HCFU) is more active against various tumors in mice and less toxic to host animals than its parent drug 5-fluorouracil (5FU). Iigo et al. (1978) have tested the activities of these drugs on Lewis lung carcinoma and B16
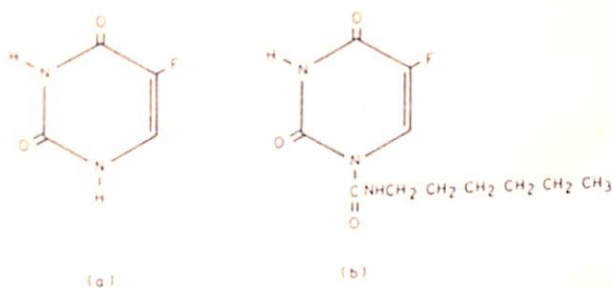
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Fig. 1. Chemical structures of (a) 5-fluorouracil and (b) 1-hexylcarbamoyl-5-fluorouracil.

melanoma. It is evident from the structure of two drugs (Fig. 1) that HCFU will be more hydrophobic and more surface active than its parent compound 5FU. Prompted by this clue, 5FU and two of its derivatives, HCFU and 1-(2-tetrahydrofuryl) 5-fluorouracil (FT), have been investigated for the contribution of liquid membrane phenomena to their action, the results are reported in this communication.

All the 3 drugs, 5FU, HCFU and FT, have been shown to generate liquid membranes in series with a supporting membrane. Transport of relevant permeants through the liquid membranes generated by these drugs in series with the supporting membrane has been studied. The data obtained from these model experiments indicate that the modification in the transport of relevant permeants due to the drug liquid membranes likely to be generated at the sites of action may also make a significant contribution to the biological actions of these drugs. In these studies a non-specific non-living membrane has been chosen deliberately as the supporting membrane for the liquid membranes. Thus the possibility of active and specific interactions of these drugs with the constituents of biomembranes as cause for modification in the transport of relevant permeants is totally ruled out. The role of passive transport through the liquid membranes in the action of these drugs is highlighted.

Materials and Methods

Materials

HCFU, 5FU and FT, (all from Mitsui Phar-

maceuticals, Inc., Tokyo, Japan); aspartic acid, folic acid, glutamine and glycine (all from Loba-Chemie) and cyanocobalamin (Sigma V-2876) were used in the present experiments. All other chemicals were analytical grade reagents. Distilled water, distilled twice in an all-pyrex glass still, was used for preparing the solutions. Aqueous solutions of HCFU and FT, which are not so easily soluble in water, were prepared by adding to the aqueous phase, with vigorous stirring, the necessary volume of ethanolic stock solution of known concentration of these substances. In the aqueous solutions of HCFU and FT thus prepared the final concentration of ethanol was never allowed to exceed 0.1% (v/v). It was experimentally found that 0.1% solution of ethanol in water did not lower the surface tension of water to any measurable extent.

Methods

The critical micelle concentrations (CMCs) of 5FU, HCFU and FT were estimated from the variation of surface tension with concentration and are recorded in Table 1. The surface tensions were measured using a Fisher tensiomat model 21.

The all glass cell described earlier (Tandon et al., 1986) was used for the transport studies. It is reproduced in Fig. 2 for ready reference. It essentially consisted of two compartments C and D separated by a Sartorius cellulose acetate microfiltration membrane (Cat no. 11107, pore size 0.2 μm) of thickness 1×10^{-4} m and area 2.55×10^{-5} m^2 which acted as a supporting membrane for the liquid membranes. To obtain the hydraulic permeability data which were utilised to demonstrate the formation of liquid membrane in series with the supporting membrane, aqueous solutions of varying concentrations of the drugs were filled in

TABLE 1

Critical micelle concentrations (CMC) of anticancer drugs

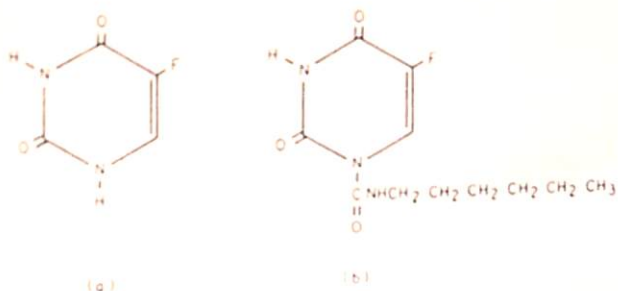| Drugs	CMC (M)
5FU	8.0×10^{-10}
FT	7.5×10^{-11}
HCFU	6.1×10^{-11}

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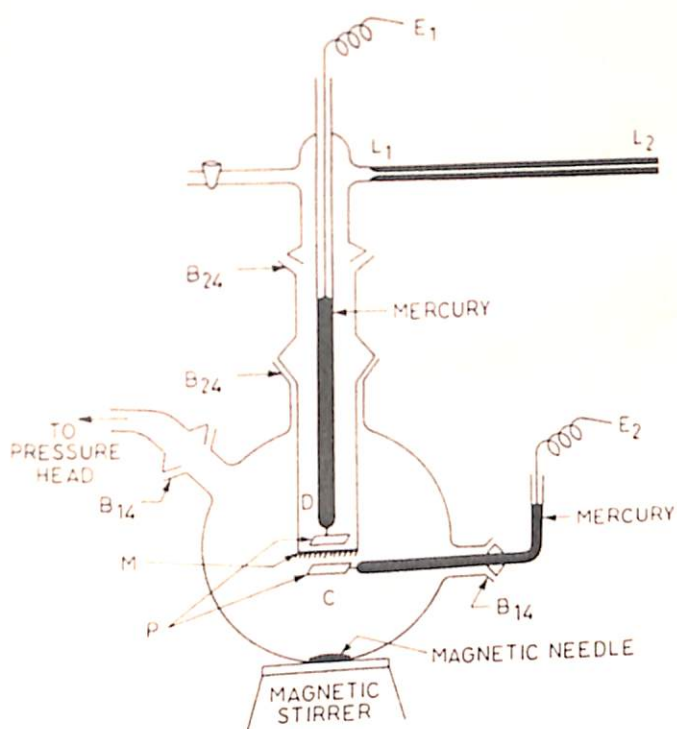| Drugs	CMC (M)
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Fig. 2. Transport cell. M, supporting membrane, P, bright platinum electrodes, E₁ and E₂ electrode terminals, L₁L₂, capillary.

described in the publications cited above. For solute permeability measurements two sets of experiments were performed. In the first set of experiments an aqueous solution of the drug was filled in compartment C along with the permeant and compartment D was filled with distilled water (Fig. 2). In the second set of experiments the aqueous solution of the drug was filled in compartment D of the transport cell and compartment C was filled with the aqueous solution of the permeant. The concentrations of the drugs used in the ω measurements were always higher than their CMCs.

All measurements were made at constant temperature using a thermostat set at $37 \pm 0.1°C$.

Estimations

The amounts of the various permeants transported to compartment D were estimated as follows:

Folic Acid. The amount of folic acid was estimated using a Cary 17-D spectrophotometer by measurement of ultraviolet absorption at 283 nm —the absorption maximum of folic acid in 0.1 M sodium hydroxide solution (Hashmi, 1973a).

Cyanocobalamin. The amount of cyanocobalamin was estimated spectrophotometrically in an aqueous solution by measurement of absorbance at 361 nm (Hashmi, 1973b; Heller, 1980).

Amino acids. The amounts of glycine, glutamine and aspartic acid were estimated from the amount of their reaction products with ninhydrin measured at 570 nm (Moore and Stien, 1954).

compartment C of the transport cell (Fig. 2) and compartment D was filled with distilled water. The concentrations of the drugs chosen were such that the hydraulic permeability data were obtained both below and above the CMCs of the drugs. The details of the method used for the hydraulic permeability measurements have been described earlier (Bhise et al., 1984a and b, 1985a and b; Srivastava et al. 1984, 1985, 1986; Tandon et al. 1986).

The solute permeability (ω) of the relevant permeants in the presence of the liquid membranes generated by the drugs were estimated using the equation (Katchalsky and Curran, 1967)

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

where J_s stands for the solute flux per unit area of the membrane, $\Delta\pi$ is the osmotic pressure difference across the membrane and J_v is the volume flux. The method of measurement was the same as

Results and Discussion

The hydraulic permeability data at various drug concentrations in the case of all three drugs, were found to be in accordance with the proportional relationship

$$J_v = L_p \Delta p \qquad (2)$$

where J_v represents the volume flux per unit area of the membrane, L_p the hydraulic conductivity

TABLE 2

Values of L_p at various concentrations of 5FU, FT and HCFU

	Conc. ($\times 10^{11}$ M)	$L_p \times 10^8$ (m^{3}s^{-1}N^{-1}) *	$L_p \times 10^8$ (m^{3}s^{-1}N^{-1}) **
5FU	0.000	—	2.162 ± 0.064–
	20.00 (0.25 CMC)	1.930 ± 0.058	1.944 ± 0.056
	40.00 (0.5 CMC)	1.720 ± 0.054	1.726 ± 0.059
	60.00 (0.75 CMC)	1.573 ± 0.074	1.508 ± 0.041
	80.00 (CMC)	1.290 ± 0.034	–
	160.00	1.260 ± 0.061	
	240.00	1.266 ± 0.064	
FT	0.000	2.162 ± 0.064	
	1.875 (0.25 CMC)	1.778 ± 0.086	1.805 ± 0.081
	3.750 (0.5 CMC)	1.418 ± 0.049	1.406 ± 0.115
	5.625 (0.75 CMC)	1.095 ± 0.059	1.106 ± 0.039
	7.500 (CMC)	0.755 ± 0.017	–
	15.000	0.751 ± 0.025	
	22.500	0.761 ± 0.031	
HCFU	0.000	2.162 ± 0.064	
	1.525 (0.25 CMC)	1.795 ± 0.041	1.770 ± 0.049
	3.050 (0.5 CMC)	1.422 ± 0.030	1.377 ± 0.036
	4.575 (0.75 CMC)	0.999 ± 0.018	0.985 ± 0.023
	6.100 (CMC)	0.592 ± 0.010	–
	12.200	0.594 ± 0.006	
	18.300	0.582 ± 0.018	

The values reported for L_p are arithmetic mean of 10 repeats ± S.D.
* Experimental values.
** Calculated values using mosaic model.

coefficient and Δp the applied pressure difference across the membrane. The values of L_p estimated from the slopes of J_v versus Δp plots, in the case of all 3 drugs, show a progressive decrease with increase in the concentrations of the drugs (Table 2) upto the CMCs of the drugs beyond which they become more or less constant. This trend in the values of L_p is in keeping with Kesting's liquid membrane hypothesis (Kesting et al., 1968) according to which, when a surfactant is added to an aqueous phase, the surfactant layer which forms spontaneously at the interface acts as a liquid membrane and modifies the mass transfer across the interface. As the 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. Analysis of the values of L_p in the light of the mosaic model (Spiegler and Kedem, 1966; Sherwood et al., 1967; Harris et al., 1976) fur-

nishes additional evidence in favour of the formation of liquid membranes by the drugs in series with the supporting membrane. Following the arguments, based on the concept of the progressive coverage, given in earlier publications (Bhise et al. 1984a and b, 1985a and b; Srivastava et al., 1985, 1986; Tandon et al. 1986), it can be shown that at concentrations less than the CMCs of the surfactants the value of L_p should be equal to $(1 - n)L_p^c + nL_p^s]$ where $n \leq 1$ and the superscripts c and s represent the values of L_p at 0 and the CMC of the surfactant, respectively. The values of L_p thus computed at several concentrations of the drugs below their CMCs compare favourably with corresponding experimental values in the case of all the 3 fluorouracils (Table 2).

Solute permeability data

Since all 3 drugs, being surface-active in nature, have both hydrophilic and hydrophobic parts in

their structure, it is expected that the hydrophobic ends of the drugs molecules in the liquid membrane would be preferentially oriented towards the hydrophobic supporting membrane and the hydrophilic moieties would be drawn outwards away from it. Thus in the first set of solute permeability experiments the permeants would face the hydrophilic surface of the drug liquid membrane generated in series with the supporting membrane while in the second set of experiments they would face the hydrophobic surface. The data on the solute permeability of relevant permeants in the two orientations of the drug molecules in the liquid membranes are recorded in Table 3 along with the corresponding values from control experiments where no drug was used.

Antimetabolites in general are known to act by impairing the synthesis of purine and pyrimidine bases by interfering with folic acid metabolism or prevent the incorporation of the bases into nucleic acids (Bowman and Rand, 1980a). The steps involved are known to be enzyme-catalysed. For example 5FU is ultimately converted enzymatically into 5-fluorodeoxyuridine-5-phosphate which inhibits the thymidylate synthetase enzyme system resulting in the blockade of DNA synthesis (Calabresi and Parks, 1980a). The present data (Table 3), however, indicate that the passive transport through the liquid membranes likely to be

generated by the fluorouracils (5FU, HCFU and FT) at the respective sites of action may also contribute to their action.

Vitamin B_{12} and folic acid, which are dietary essentials for man, are required for the synthesis of purine and pyrimidine bases and their incorporation into DNA. Their deficiency may result in defective synthesis of DNA in any cell that attempts chromosomal replication and division (Hilman, 1980). The present data indicate that the transport of both, vitamin B_{12} and folic acid, are impeded by the liquid membranes generated by all 3 drugs presently studied (Table 3). This impediment in the transport may contribute to the deficiency of vitamin B_{12} and folic acid inside the cells resulting in the defective synthesis of DNA. Thus it appears that the phenomenon of liquid membrane formation may also contribute to the anticancer activities of 5FU and its derivatives. A perusal of Table 3 further reveals that inhibition in the transport vitamin B_{12} and folic acid, is more when the permeants face the hydrophilic surface of the liquid membranes than when they face the hydrophobic surface. This observation indicates that the specific orientation of the drug molecules in the liquid membranes with their hydrophilic ends facing the permeants may be necessary on cancerous cells while the drug molecules in the liquid membranes on the normal cells may have

TABLE 3

Solute permeability (ω) of various permeants in the presence of 5FU, FT and HCFU

Permeant	Initial concentration (mg/liter)	Control $\omega \times 10^9$	5FU $(1 \times 10^{-9}$ M) $\omega \times 10^9$ C	5FU $(1 \times 10^{-9}$ M) $\omega \times 10^9$ D	FT $(1 \times 10^{-10}$ M) $\omega \times 10^9$ C	FT $(1 \times 10^{-10}$ M) $\omega \times 10^9$ D	HCFU $(1 \times 10^{-10}$ M) $\omega \times 10^9$ C	HCFU $(1 \times 10^{-10}$ M) $\omega \times 10^9$ D
Aspartic acid	150	0.856 ±0.011	0.628 ±0.004	0.688 ±0.006	0.475 ±0.020	0.715 ±0.062	0.398 ±0.008	0.568 ±0.042
Cyanocobalamin	30	0.488 ±0.018	0.281 ±0.024	0.365 ±0.021	0.316 ±0.015	0.379 ±0.018	0.282 ±0.026	0.347 ±0.037
Folic acid	0.05	8.715 ±0.266	6.013 ±0.557	7.541 ±0.316	6.631 ±0.496	7.590 ±0.010	4.406 ±0.220	5.743 ±0.334
Glutamine	500	0.474 ±0.031	0.759 ±0.065	0.694 ±0.052	0.160 ±0.011	0.363 ±0.014	0.417 ±0.013	0.399 ±0.008
Glycine	100	0.265 ±0.010	0.412 ±0.055	0.644 ±0.168	0.151 ±0.002	0.182 ±0.003	0.181 ±0.001	0.195 ±0.004

Values of ω are repored as arithmetic mean of 10 repeats ±S.D., in mol·s^{-1} N^{-1}. C, drug in compartment C; D, drug in compartment D.

the other orientation - hydrophobic ends facing the permeants. This inference in turn implies that surface of the membranes of the cancerous cells should be less hydrophilic than those of the normal cells. Though there are some indications in literature (Bowman and Rand, 1980b; Burger 1971; Emmelot, 1964a) that the neoplastic state may also arise through an alteration in the surface properties of the cells, a thorough probe in terms of hydrophilicity of the cell surface is called for to substantiate this conjecture.

Amino acids like glycine, glutamine and aspartic acid are also required, in addition to folic acid, for the purine ring synthesis (Bowman and Rand, 1980c; Emmelot, 1964b). Compounds from which the atoms of the purine ring are derived in the biosynthetic pathway are depicted in Fig. 3. The present data (Table 3) indicate that except in the case of 5FU, the transport of glycine, glutamine and aspartic acid is also impeded in addition to folic acid and vitamin B_{12}, by the liquid membranes generated by both FT and HCFU. In the case of 5FU the transport of glycine and glutamine was enhanced. The impediment in the transport of the amino acids also in the case of HCFU and FT, was more in the specific orientation of the drug molecules in the liquid membrane with their hydrophilic ends facing the permeants. This impediment in the transport observed in the case of FT and HCFU may also be a factor responsible for the impairment of the synthesis of purine bases contributing to the anticancer activity of these drugs.

It has been reported by Iigo et al. (1978) that of the 3 drugs, HCFU, FT and 5FU, HCFU is most potent. This finding is consistent with the liquid membrane hypothesis for drug action (Srivastava et al., 1984). The CMC of HCFU is the lowest (Table 1). As CMC is the concentration at which a complete liquid membrane is generated at the interface, it would appear that of the three drugs, HCFU would require the lowest concentration for the development of a complete liquid membrane at the site of action. Since modification of the transport of the relevant permeants, which affects the biological effect, is maximum when a complete liquid membrane is generated, the concentration of HCFU required to produce the maximum biological effect would be the lowest amongst the 3 drugs, making HCFU the most potent drug.

Some of the adverse side effects of cytotoxic drugs include megaloblastic anaemia (Horler, 1981), neurological disorders relating to spinal column and cerebral cortex (Calabresi and Parks, 1980b), ineffective haematopoiesis and pancytopenia (Bowman and Rand, 1980d). These symptoms are also the symptoms of deficiencies of vitamin B_{12} or folic acid or both (Hilman, 1980; Finch et al. 1956; Stebbins et al. 1973; Stebbins and Bertino, 1976). The impediment in the transport of vitamin B_{12} and folic acid in the specific orientation of the drug molecules in the liquid membrane with their hydrophobic ends facing the permeants, which may be the orientation on the normal cells, could also be a plausible explanation for the reported side-effects.
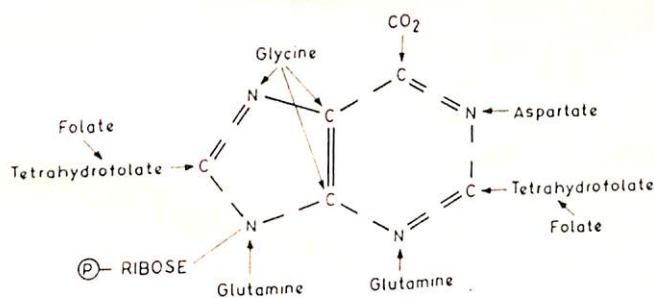
Fig. 3. Compounds from which the atoms of the purine ring are derived in the biosynthetic pathway. The breaks in the bonds separate the groups of atoms derived from each source (Bowman and Rand, 1980c).

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