CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Studying the molecular events occurring on cell membranes, as well as the multiplicity of interactions with bioactive compounds, in either physiological or pathological situations, is of paramount importance, to enlarge our knowledge of many diseases and to identify further potential therapeutic targets.

1.1 Cell and Cell Membrane

Cell is the basic structural and functional unit of all the living organisms. It is the smallest unit of life that is classified as a living thing (except virus, which consists only from DNA/RNA covered by protein and lipids), and is often called the building block of life. The cytoplasm of a cell is surrounded by a cell membrane or plasma membrane.

The cell membrane is a biological membrane which surrounds the cytoplasm of living cells. It physically separates the intracellular components from the extracellular environment. Cell membranesare selectively permeable to ions and organic molecules and control the movement of substances in and out of cells. The basic function of the cell membrane is to protect the cells from hostile surroundings.

The plasma membrane serves as an interface between the interior and exterior of the cell and has definite physical and chemical structure and has an inherent property of regulating the flow of material into and out of the cell. It consists of the lipid bilayer with embedded proteins. Cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signaling and serve as the attachment surface for several extracellular structures and intracellular cytoskeleton.

The first living cell probably came into being when a membrane was formed, separating that cell's previous contents from the rest of the universe[18]. A cell which is in equilibrium with its surrounding is dead, and it happens when the plasma membrane is not able to discriminate between the intracellular and intercellular species[19]. Membranes describe the external boundary of cells and regulate molecular traffic across that boundary[20]. Though the functions of various cells and the sub-cellular organelles vary widely, they all have a common structure of the membrane composed of lipids and proteins.

1.2 STAGES IN THE EVOLUTION OF STRUCTURE OF BIOMEMBRANES

The presence of cytoplasmic membrane determining the volume of a cell was postulated a long time back. Towards the end of 19th century, utilizing red blood cells as osmometer, Overton found that there exist a close connection between the capability of molecules to penetrate into cells and

their solubility in hydrophobic media (Olive oil). On this basis, he suggested that the cell was covered with an invisible and very thin layer of fats[1, 21]. Later, the study of composite arrangement and functions of the membrane components has led to the development of a specialized field called 'membranology'.

The measurement of properties of membranes such as electrical capacitance, resistance, surface tension etc., led to the conclusion that the membrane consists mainly of lipids. In 1925, Gorter and Grendel resolved that the plasma membrane contains lipids in the form of a bilayer. Later in 1935, Danielle and Davson proposed a model for biomembranes, which constitute a double layer of phospholipids and polar head groups to facilitate transport of ions and polar molecules. They proposed that the polar head groups of lipid molecules are facing out and a coat of proteins is present over these polar groups to facilitate transport of ions and molecules. In 1972, Singer and Nicholson proposed the widely accepted fluid-mosaic model for the cellular membrane[18, 20] and [21]. This model proposes the presence of a bilayer of lipid molecules and proteins embedded either throughout the membrane or on the periphery of the membrane. Figure 1.1 shows the Fluid (lipids) - Mosaic (proteins) model structure of biological membranes.

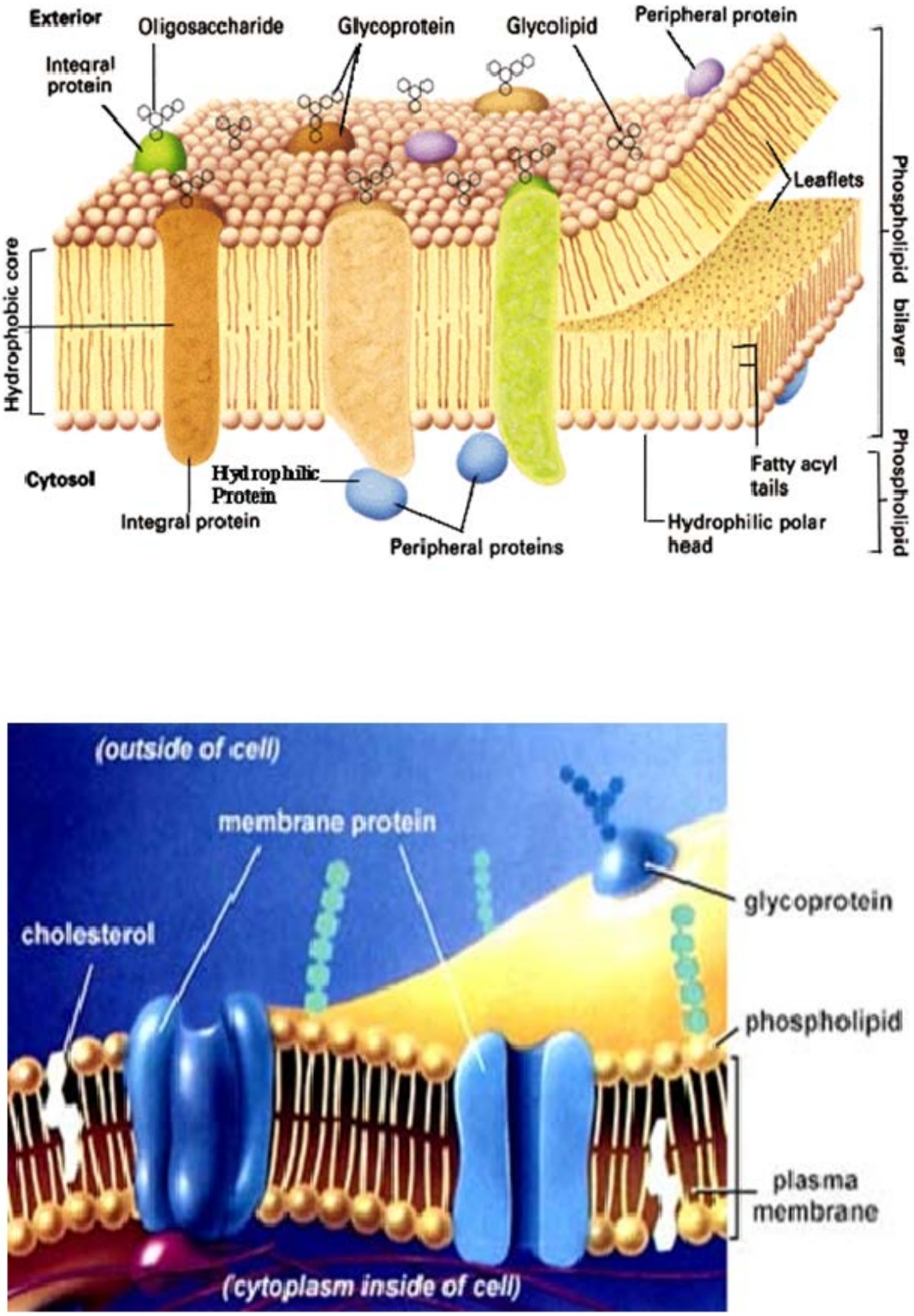


Figure 1.1 Fluid-Mosaic model of biological membrane.

1.3 FUNCTIONS OF CELL MEMBRANES

The cell membrane is an important part of the cell and performs many functions. Some important functions of the cell membrane are listed below[1-4].

1. They organize complex reaction sequences.
2. They are central to both biological energy conversions and cell to cell communications.
3. They are capable of sealing temporary breaks, which facilitate cell fissions and cell fusions.
4. Membranes are not merely passive barriers. They exhibit selective permeability. They restrict the entry of some species and allow some other unimpeded.
5. They include an array of proteins specialized for promoting or catalyzing a variety of molecular events.
6. They bring out active transport against both concentration and electrical potential gradients. They pump more specific organic solutes and inorganic ions across the membranes against concentration gradients. For e.g. Na^+ ions are constantly pumped out of the cell while K^+ ions are pumped in.
7. Receptors on the plasma membrane help in the transmission of information from and into the cell. These receptors are very sensitive to

extremely low concentrations of substances such as drugs present in the environment.

8. They sense extra-cellular signals, and the energy transducers convert one form of energy into another.

The protein and lipid compositions of the cell membrane depend upon its specialized functions. The mitochondrial membrane is rich in proteins and enzymes to carryout oxidation-reduction reactions to generate energy[3, 4]. The nerve cell membrane, on the other hand, has lesser amounts of proteins as it is mainly involved in electrical signal conduction. Though the amounts of proteins present and the functions of membranes vary with the cell, the bilayer leaflet of lipids is a common feature among all these membranes.

1.4 MOLECULAR MAKEUP OF BIOMEMBRANE

The biomembranes are composed of lipids, proteins, cholesterol and small amounts of carbohydrates. Different membranes have different compositions of these constitutions and there is a difference in the composition of lipids also[1]. The protein: lipid ratio varies greatly with the type of membranes.

- The inner mitochondrial membrane contains about 76% protein.
- The myelin membrane contains only 18% protein.

1.5 PHOSPHOLIPID MOLECULES AND THEIR SELF ORGANIZATION

Phospholipid molecules are amphiphilic molecules containing both hydrophilic and hydrophobic sections with glycerol backbone. They originate from glycerol, where two of three alcoholic groups, on successive carbon atoms, are esterified with two long chain saturated or unsaturated fatty acids and third alcoholic group is esterified with phosphoric acid. This structure is known as phosphatidic acid. When the phosphate groups in the phosphatidic acid are connected with a nitrogen base such as serine, choline and ethanolamine, the resulting structure is called phospholipid.

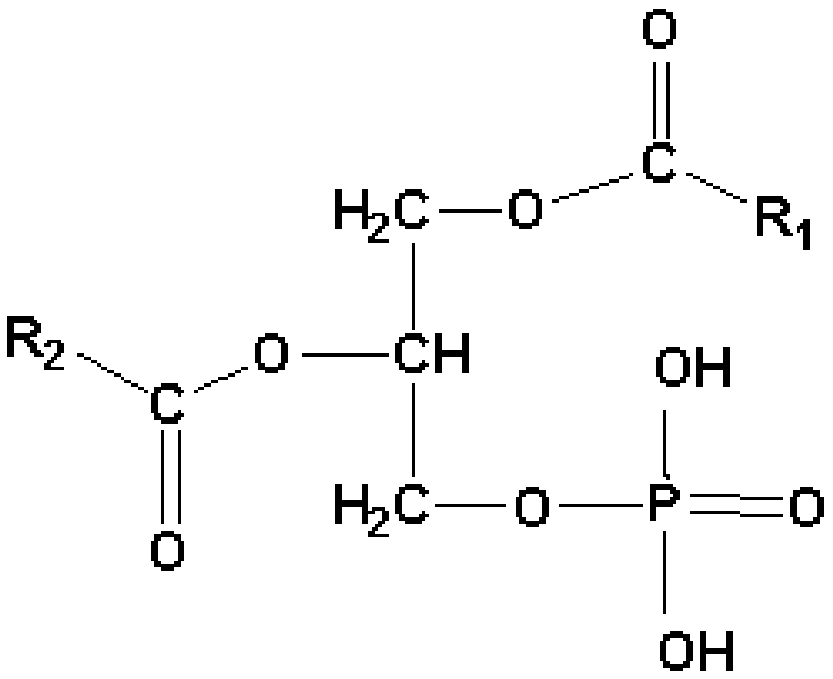
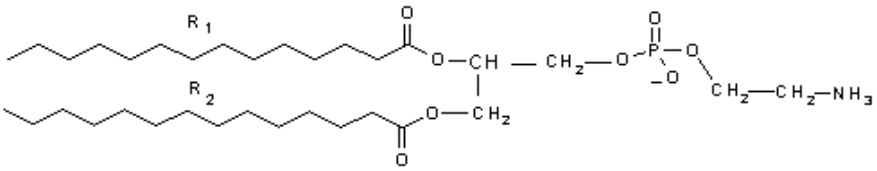
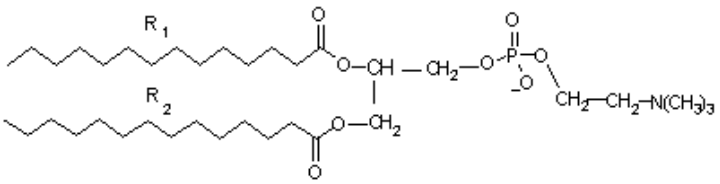


Figure 1.2 Structure of Phosphatidic acid

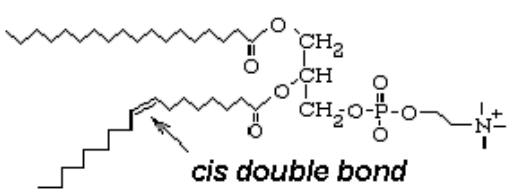
R_1 and R_2 represent two long hydrocarbon chins in the fatty acids. The most common phospholipids are phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), and phosphatidylinositol (PI). These are the derivatives of phosphatidic acid. The structures of phosphatidic acid, PC and PE etc., are given in Figure 1.3.

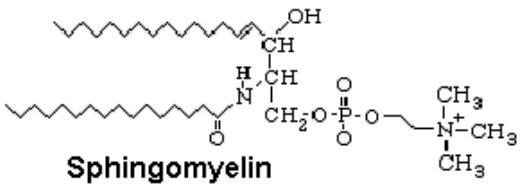


Phosphatidyl ethanolamine

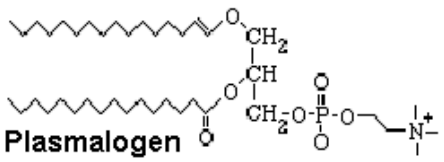


Phosphatidyl choline

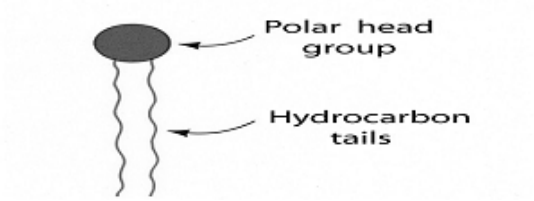




Sphingomyelin



Plasmalogen



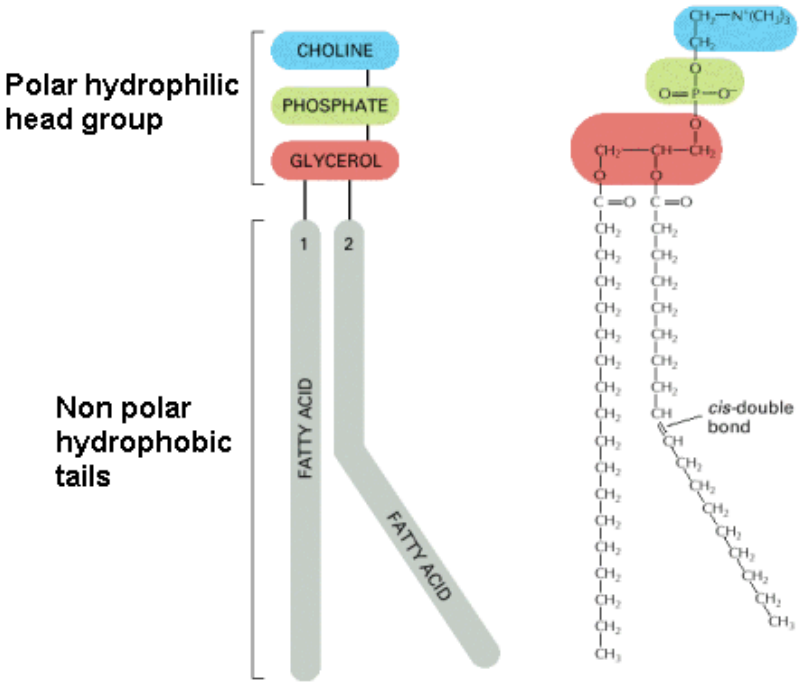


Figure 1.3 Structure of Phospholipid molecules.

In a phospholipid molecule, the hydrophobic nature is derived from non-polar fatty acid chains and the polarity is derived from a negatively charged phosphate group and sometimes by a positively charged amino group. Nearly all fatty acyl chains found in the membranes of eukaryotic cells have an even number of carbon atoms (usually 16, 18, or 20). Unsaturated fatty *acyl* chains normally have one double bond but some have two, three or four. In general, all such double bonds are of cis configuration. A cis double bond introduces a rigid kink in the otherwise flexible straight chain of a fatty acid.The possible fatty acid chains in phospholipids and parent nitrogen basesare given in Table 1.1 and 1.2 respectively.

Table 1.1

Common fatty acids present in phospholipid molecules

No. of carbon atoms	No. of double bonds	Common name	IUPAC name	Molecular formula
12	0	Laurate	Dodecanoate	$CH_3(CH_2)_{10}COO^-$
14	0	Myristate	Tetradecanoate	$CH_3(CH_2)_{12}COO^-$
16	0	Palmitate	Hexadecanoate	$CH_3(CH_2)_{14}COO^-$
18	0	Stearate	Octadecanoate	$CH_3(CH_2)_{16}COO^-$
20	0	Arachidate	Eicosanoate	$CH_3(CH_2)_{18}COO^-$
22	0	Behenate	Docosanoate	$CH_3(CH_2)_{20}COO^-$
24	0	Lignocerate	Tetracosanoate	$CH_3(CH_2)_{22}COO^-$
16	1	Palmitotate	*Cis*Δ^9-Hexadecanoate	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COO^-$
18	1	Oleate	*Cis*Δ^9-Octadecanoate	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COO^-$
18	2	Linoleate	*Cis,cis*$\Delta^{9,12}$ Octadecatrienoate	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COO$-
18	3	Linoleate	All *cis*$\Delta^{9,12,15}$-Octadecatrienoate	$CH_3(CH_2)_4(CH{=}CHCH_2)_3(CH_2)_6COO^-$
20	4	Arachidonate	All *cis*$\Delta^{5,18,11,14}$-Eicosaretraenoate	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COO^-$

Table 1.2

Examples of some phospholipids and their parent nitrogenous bases

Name of the nitrogen base	Formula of nitrogen base	Name of the Phospholipid
Ethanolamine	$-CH_2CH_2NH^{3+}$	Phosphatidylehanolamine
Choline	$-CH_2CH_2N(CH_3)^{3+}$	Phosphatidylcholine (Lecithin)
Serine	$- CH_2CH(NH_3)COO^-$	Phosphatidylserine

A polar head group and two hydrophobic tails give an asymmetric structure to phospholipid molecules (Fadeel, B. and Xue, D., 2009). This asymmetrical design makes these molecules perfectly suited to self-assemble themselves in solution into organized structures. The polar part of a phospholipid easily makes physical bonds with water dipoles and/or ions present in the aqueous solution. On contrary, the hydrophobic tails can make only weak hydrophobic bondings with other fatty acid chains.

On the basis of these simple considerations, three major space organizations of phospholipid assemblies can be qualitatively predicted (Massimo Grattarola and Giuseppe Massobrio, 1998).

1. An assembly of gently dissolved phospholipid molecules on the surface of an aqueous solution will tend to self organize into a monolayer with head groups interacting with the solution and all the tails parallel to each other out in gas phase on the top of the solution. This can be considered

as an example of molecular insulator with few nanometers (2 to 3 nm) thickness, which can be deposited on the surface of a solid material appropriately dipped into the solution.

2. An assembly of forced phospholipid molecules inside an aqueous solution will tend to self-assemble into small(4 to 6 nm) drops, with the external surface formed by the polar head groups and the core by packed, water excluding, hydrophobic tails. This self-organized structure is called micelle.
3. A third way of satisfying the hydrophilic/hydrophobic rule is a self – sealing spheroidal bilayer. This is a more complex system which can separate an outer aqueous solution from an inner aqueous solution, with two polar surfaces facing the two aqueous solutions. The resulting structute is known as liposome. Liposomes are of relevance for at least two reasons: First, biocompatible liposomes can be loaded with drugs and then injected into the blood. In this way the drug can be released inside the body through the slowly leaking bilayer of the liposome with a time scale that can be, to some extent, programmed by experimenter. Second, for the purposes of forming artificial cell membranes to understand and study the functions of cell membranes.

Three self organized structures of phospholipid molecules ae sketched in Figure.1.4

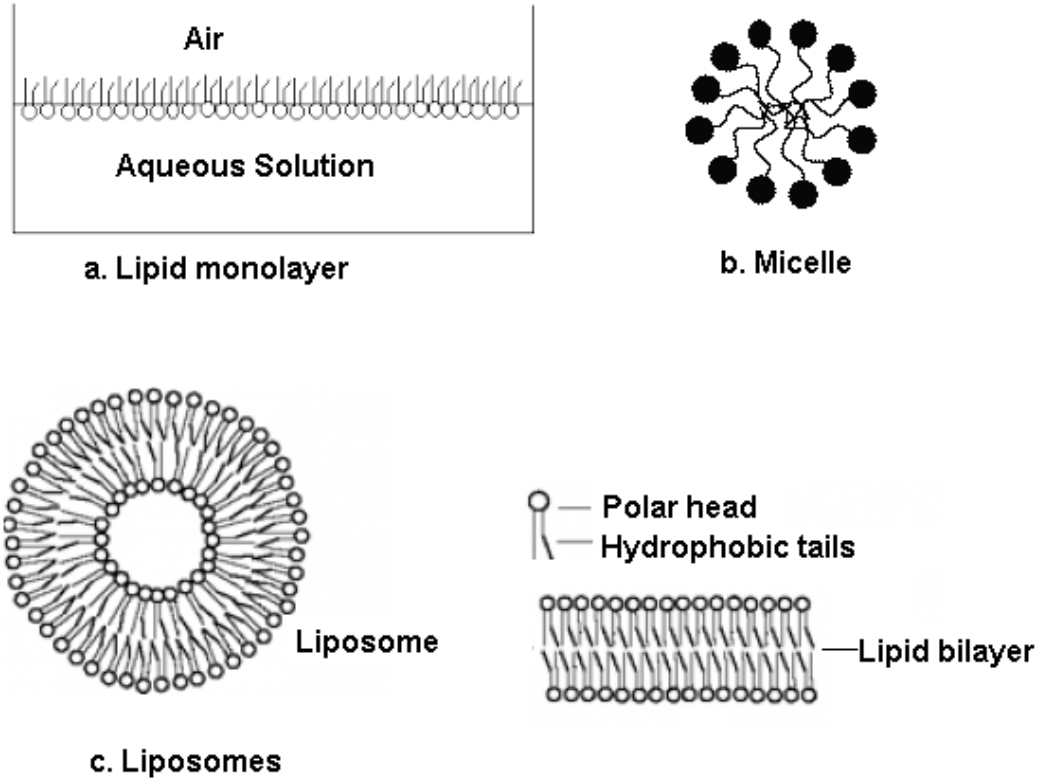


Figure.1.4 Self Organized structures of phospholipid molecules

The life of living organisms depends on the coordinated activity of many interconnected parts of body on different levels. The fundamental structural unit of these parts is the cell, which is basically formed by the cytoplasm, a fluid matrix in which nucleus and cell organelles are suspended. The cytoplasm is separated from its surroundings a bilayer wall called cell membrane. Additionally the cell membranes contain a wide variety of biomolecules, primarily proteins and lipids, which take part in many cellular processesh as ion channels, conductance, cell snalling etc (Navratil et al, 2010). The bilayer wall is made of mostly phospholipid molecules (Navratil et al, 2010).In biological systems, the cell membranes, also known as plasma membranes, play a key role in the selective transport of molecules, receptor binding, enzymatic activity and control of cell to cell interactions.

1.6 MEMBRANE PROTEINS AND THEIR FUNCTIONS

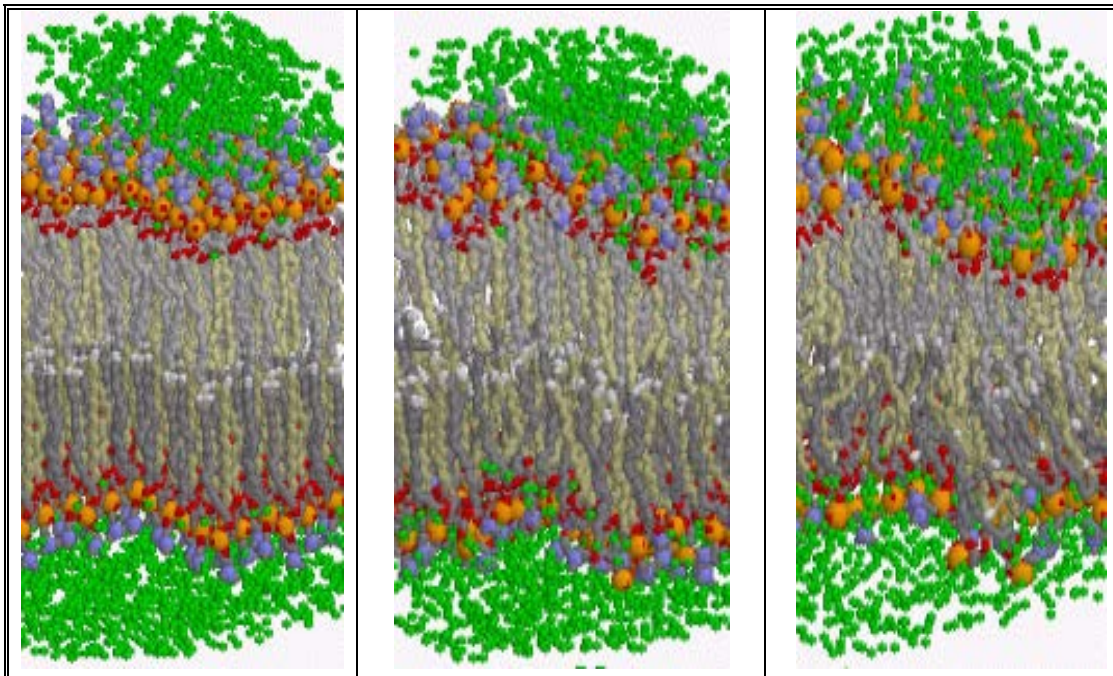
There are different kinds of proteins in cell membranes to carryout varied functions. They are classified as:

1. Transmembrane proteins: They are also referred to as integral or intrinsic proteins, which span the bilayer completely. These proteins function as channels and carriers, which are associated with the transport of ions and molecules. They interact with the bilayer by hydrophobic forces and with head groups with hydrophilic forces. These proteins cannot easily be dislodged from the bilayer unless the bilayer is disrupted.

2. Peripheral proteins: They are also called as extrinsic proteins, which are immersed partially in the lipid matrix. They are attached to the head groups of the lipids or to the superficial portions of the integral proteins. They can easily be removed from the membrane by mild treatments.
3. Proteins absorbed onto the surface of the membrane (typically enzymes).

1.7 FLUIDITY OF LIPIDS IN MEMBRANES

The different states (conformations) of lipids in bilayer membranes and consequences to the structure are shown in Figure 1.5.The bilayer structure has ‘fluidity’. The membrane is a liquid crystal in which lipids and proteins diffuse within the plane of the membrane. The very function of proteins and enzymes is modulated by the membrane micro-fluidity also. The membrane lipids are in constant motions because of the fluidity of membrane. A single phospholipid molecule may circumnavigate the cell in a few seconds. Any change brought about in the fluidity of membrane lipids would result in corresponding conformational changes in proteins and in lipid-lipid, lipid-protein and protein-protein interactions. The alterations in the fluidity of membranes and changes in the conformations of proteins form the basis for many drug-membrane interaction studies.



Molecular Dynamic Simulation of Phosphatidylcholine Bilayer

Carbon/Palmitic/ Oleic acid/Water Oxygens Nitrogen Phosphorus Oxygen

(Heller et al,1993)

Figure 1.5 Structure of the bilayer affected by conformational changes and fluidity in lipid molecules.

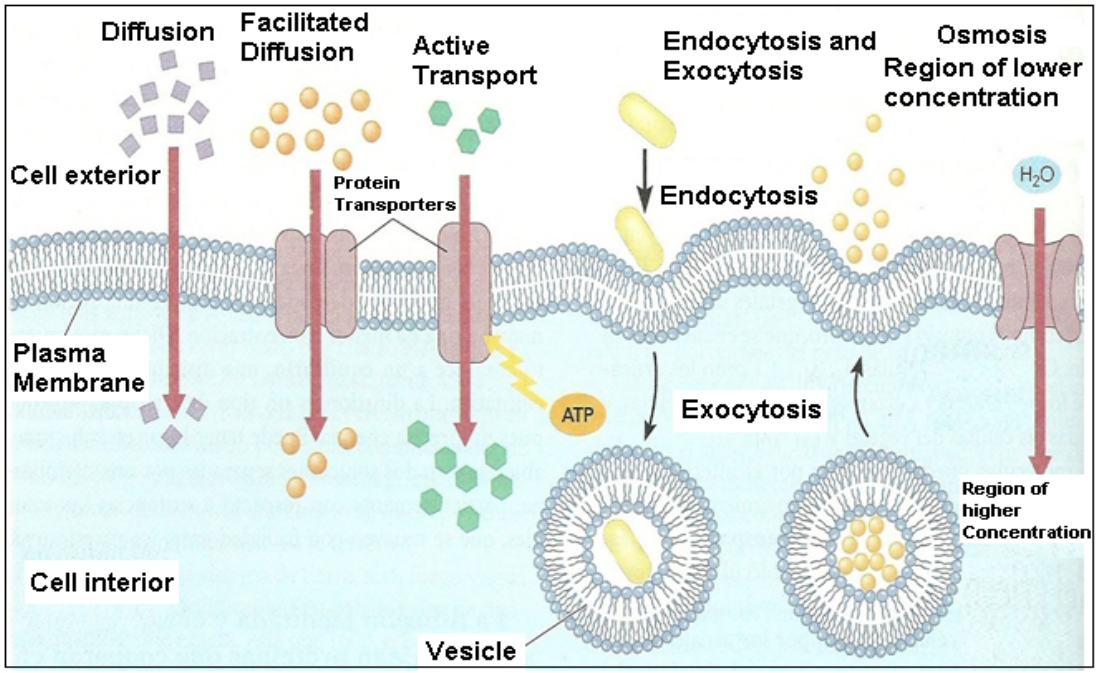
1.8 TRANSPORT OF MOLECULES THROUGH CELL MEMBRANES

The movement of substances across the membrane can be either "passive", occurring without the input of cellular energy, or active, requiring the cell to expend energy in transporting it. The membrane also maintains the cell potential. The cell membrane thus works as a selective filter that allows only certain things to come inside or go outside the cell. The cell employs a number of transport mechanisms that involve biological membranes:

1. Passive diffusion and osmosis: Some substances (small molecules, ions) such as carbon dioxide (CO_2), oxygen (O_2), and water, can move across the plasma membrane by diffusion, which is a passive transport process. Because the membrane acts as a barrier for certain molecules and ions, they can occur in different concentrations on the two sides of the membrane. Such a concentration gradient across a semi-permeable membrane sets up an osmotic flow for the water.
2. Transmembrane protein channels and transporters: Nutrients, such as sugars or amino acids, must enter the cell, and certain products of metabolism must leave the cell. Such molecules are pumped across the membrane by transmembrane transporters or diffuse through protein channels. These proteins, also called permeases, are usually quite specific, recognizing and transporting only a limited food group of chemical substances, often even only a single substance.

3. Endocytosis: It is the process in which cells absorb molecules by engulfing them. The plasma membrane creates a small deformation inward, called an invagination, in which the substance to be transported is captured. The deformation then pinches off from the membrane on the inside of the cell, creating a vesicle containing the captured substance. Endocytosis is a pathway for internalizing solid particles (cell eating or phagocytosis), small molecules and ions (cell drinking or pinocytosis), and macromolecules. Endocytosis requires energy and is thus a form of active transport.

4. Exocytosis: Just as material can be brought into the cell by invagination and formation of a vesicle, the membrane of a vesicle can be fused with the plasma membrane, extruding its contents to the surrounding medium. This is the process of exocytosis. Exocytosis occurs in various cells to remove undigested residues of substances brought in by endocytosis, to secrete substances such as hormones and enzymes, and to transport a substance completely across a cellular barrier. In the process of exocytosis, the undigested waste-containing food vacuole or the secretory vesicle budded from Golgi apparatus, is first moved by cytoskeleton from the interior of the cell to the surface. The vesicle membrane comes in contact with the plasma membrane. The lipid molecules of the two bilayers rearrange themselves and the two membranes are, thus, fused. A passage is formed in the fused membrane and the vesicle discharges its contents outside the cell.



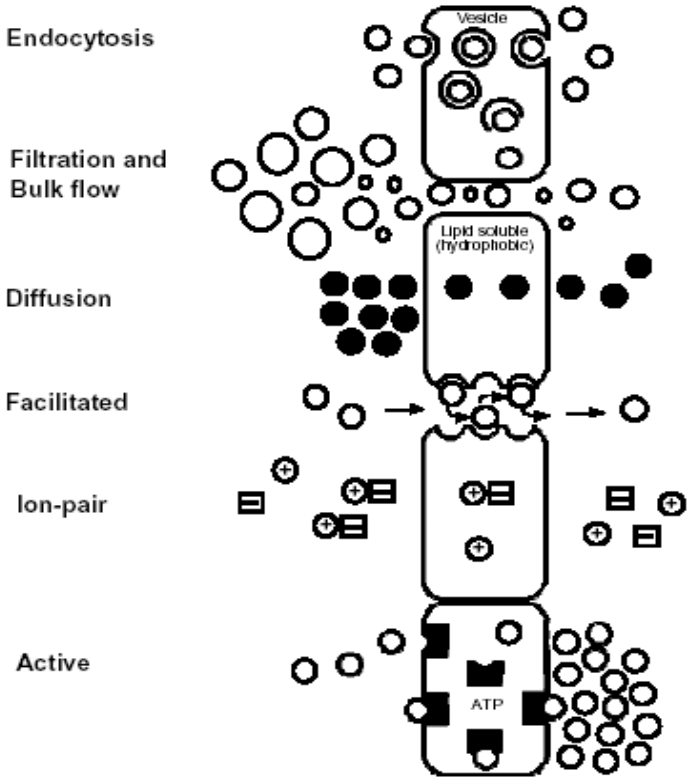


Figure 1.6 Various modes of transport of solutes (Drugs) across membranes.

The permeation of drugs via processes aqueous diffusion, facilitated diffusion (via special carriers) and pinocytosis (receptor mediated endocytosis) requires the presence of specific receptors. Each drug molecule has a unique receptor which aids its transport across the cell membrane. The receptor for a particular drug might not serve as a receptor for another. The receptor for a particular drug may not be available in all cell membranes and might be restricted to membranes in some specialized areas. Therefore, when a drug reaches the cell membrane, it gets inside the cell via specialized carriers or receptors, if available, or else, its entry into the cell depends upon its lipid solubility and its interaction with the bilayer. This gives rise to two kinds of interactions:

a. The interaction of drugs with unique proteins and receptors confirming its unique transport systems, known as specific interaction and
b. The interaction of drugs with lipid bilayer, known as non-specific interaction, which is possible in all membranes.

1.9 MODEL SYSTEMS OF THE BIOLOGICAL MEMBRANE

The complex structure of biological membranes has motivated to develop wide variety of simpler model systems whose geometry, size and composition can be tailored with great precicsion (Chan and Boxer, 2007). These artificial model membrane systems are very useful models to gain an insight into the processes occurring at the cell membrane, such as signal transduction, molecular recognition, ion transport across the membrane.

Membrane proteins or membrane active substances are also characterized by these membrane systems (Charbit et al, 2000) (Nestorovich et al, 2002) (Akeson et al, 1999). The receptor incorporated model membrane systems have a great potential deal with biosensors (Cornell et al, 2001) (Seifert et al, 1993) (Winterhalter, 1999). The most widely used model systems which stimulate the bilayer architecture are described below.

1.9.1 Liposomes

Liposomes or lipid vesicles are spherical structures with one or several lipid bilayers forming its surface. In liposomes an aqueous solution is enclosed by one or several lipid bilayers. The phospholipid molecules energy favorable structures in an aqueous solution due to the hydrophilic and hydrophobic interactions. Liposomes are classified as large multilamellar vesicles (MLV's) and small unilameller vesicles (SUV's) based on the number of bilayers and size. The structure of liposomes makes it possible either to immoblise the molecules within the membrane or encapsulate water soluble molecules in the water interior of the liposome. The choice of membrane components can modify the liposomes in desired manner and this makes them attractive model for cell membranes. In cancer treatment liposomes are also used as drug delivery systems for anti cancer agents, which increases the effectiveness and circulation time of drugs (Immordino et al, 2006) (Sushma et al, 2010) (Schroeder et al, 2012).

When phospholipids are sonicated in an aqueous solution containing the specified drug, spherical liposomes are formed incorporating the drug in their inner aqueous compartment. When injected, they travel through the blood stream and deliver drugs to the affected cells. The main trouble faced in these techniques is the degradation of phospholipids in the liver. To overcome this, the surface charges on liposomes are manipulated so that they specifically search for suitable cells in the body and fusing with the cells, deliver their drug contents into the cell. Such a concept of delivering drugs directly to specific cells is referred to as targeted delivery. Such liposomes can travel inside the body without being detected by the bodily mechanisms and are termed as stealth liposomes. Such methods are being tried to deliver anticancer drugs directly to the cancer cellsWhen an appropriate molecule is attached at the liposome surface it is possible to bind specifically to the receptor of target cells (Banerjee,2001) (Lasic, 1998).

1.10 BLACK LIPID MEMBRANES

The use of black lipid membrane as recognition element was reported first time in 1962 (Muller et al, 1962). Planar bilayer membrane reduces the chemical complexity of biological membranes. Electrical, mechanical, thermal and optical properties of these membranes can be followed through various experimental parameters such as ionic concentrations of the bath medium, ionic nature, pH, temperature, etc. Ion-transport mechanism can be easily followed in the bare membrane as well as by incorporating specific

channel forming proteins. These results help in proposing an analogous mechanism in biological membrane (Tien, 1974) (Hanke and Schlue, 1993).

The technique for the formation of BLM was developed in 1962 by Rudin, Wescott, Mueller and Tien and this method is popularly known as Rudin - Mueller method (Tien, 1974) (Hanke and Schlue, 1993). BLMs are easily formed in BLM chambers, which comprise of two aqueous compartments that are connected by an aperture of approximately 1mm diameter made in a 1mm thick PMMA or Teflon septum. When the dispersion of phospholipids (pure lipids or mixture of lipids) in a hydrocarbon solvent is applied on the aperture immersed in the aqueous bath, spontaneous thinning of the film occurs slowly with the exclusion of solvent molecules, leading to a bilayer of lipid molecules separating the two aqueous phases. Since these aqueous compartments are open and readily accessible, sampling, drug addition, medium control, measurement of electrical and mechanical properties etc., are easier. The schematic arrangement of BLM set-up is shown in Figure 1.7.

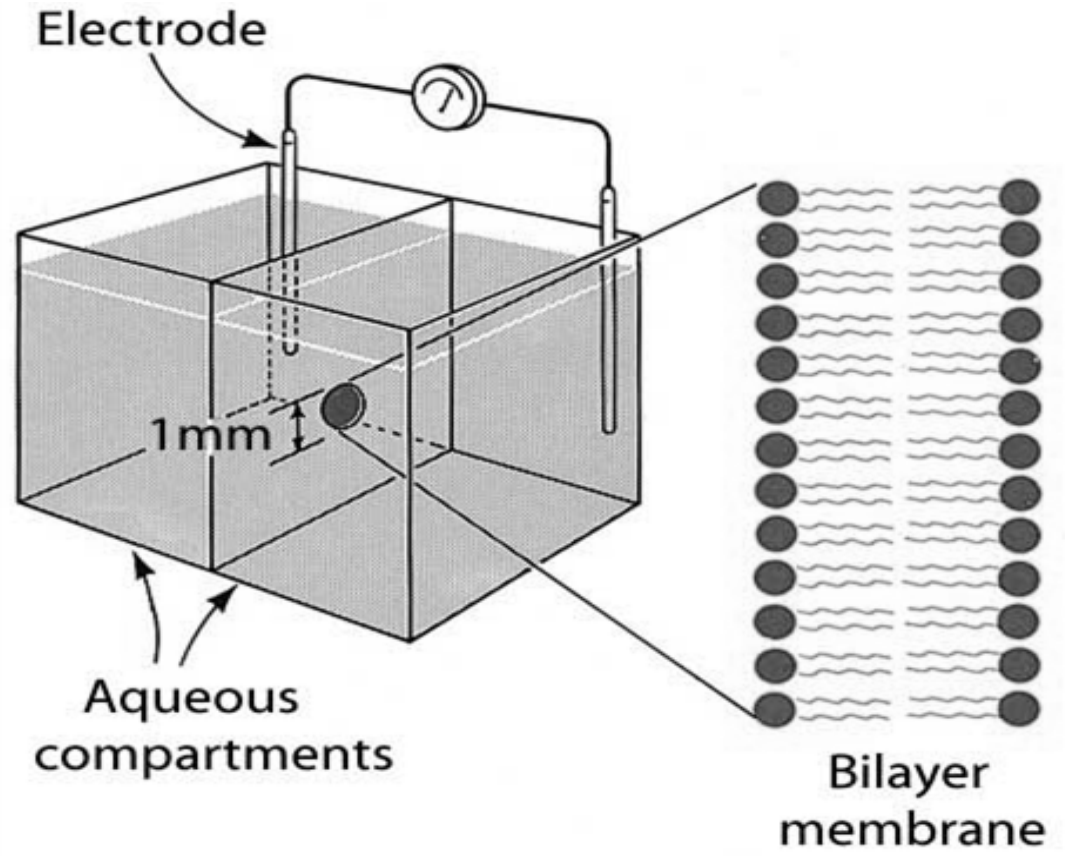


Figure 1.7 Schematic diagram of black or Planar BLM set-up.

BLM models were described as an important tool for compartmentalizing and studying the molecular interactions that occur in the biological membranes (Darold Wobschall, 1972) (Stephen et al, 1973). H.Ti. Tien, (Tien, 1974) (Hanke and Schlue, 1993), Hanke and Schlue (Hanke and Schlue, 1993) have extensively discussed the various theoretical aspects such as physical, chemical and mechanical characteristics of BLMs and practical considerations in the formation of BLMs and instrumentations. They have also described the usefulness of BLMs in studying the biomembrane events.

The thickness of BLMs and the biological membranes observed in electron microscopy are comparable and are in the order of 6-9nm and 4-13nm respectively. Capacitance values are also very similar for both the membranes. This resemblance may be considered as strong evidences in support of the bimolecular leaflet model of biological membranes. Thus, the BLM system represents the closest approach to the biological membrane ((Tien, 1974). Since natural membranes contain carriers and channels, their electrical conductance are much higher when compared to that of BLMs (Tien, 1974). Properties close to that of biological membranes can be achieved by introducing specific proteins into BLMs. To summarize:

1. Bilayer lipid membranes (BLMs) can be formed in aqueous media using a variety of lipids.
2. The intrinsic properties of unmodified BLMs are similar to those expected of an ultra thin layer of liquid hydrocarbon.

3. By introducing additives to BLM, the intrinsic or passive properties of BLM can be drastically modified. These modifiers can be selected to alter electrical, mechanical, ion selectivity, electrical excitability (or) photoelectric characteristics of the BLM.

The physical stability of planar lipid membranes is very low and much effort has been put on to improve the stability of this model membrane system (Del Castillo et al, 1966) (Ashcroft et al, 1983). The properties of BLMs such as thickness (Fettiplace et al, 1971), elasticity (Hianik et al, 1984), electrical properties (Coster and Laver, 1986) were much affected by the residues left from solvent inside the bilayer. The conduction of incorporated ion-channel is also affected (Hendry et al, 1978). Because of size limitation in this method large area BLMs can't be formed (Purrucker et al, 2001). However, the black lipid membranes are extremely valuable in the history of membrane research.

1.11 SUPPORTED BILAYER LIPID MEMBRANES (s-BLMs)

Immobilization of lipid bilayers on a solid support was considered as an alternative for BLM. These membranes were prepared on various solid surfaces i.e., glass, silicon, mica, gold or on glassy carbon electrode surfaces. Langmuir – Blodgett (LB) transfer and liposome spreading technique are the standard methods of formation of supported lipid membranes on planar solid surfaces. The attachment of bilayer membrane to planar solid surface results in a long term and highly mechanically stable model membrane system and

this is the major advantage of this method. Solid supported membranes can be accessed by a variety of sensitive surface analysis tools such as surface Plasmon resonance spectroscopy, quartz crystal microbalance, scanning probe microscopy, as well as electrochemical measurements. Thus, Supported bilayer lipid membranes overcame many of the drawbacks of black lipid membranes, namely the fragility and sensitivity to electrical and mechanical disturbances (Phung et al, 2011) (Tien et al, 1989) (Sabo et al, 1997) and represent a useful model system to study the basic interactions in biological cell membranes and are of great interest for technological applications such as biosensors and molecular electronic devices (Bordi et al, 2002) (Sackmann, 1996) (Steinem et al, 1996) (Gu et al 1996) (Raguse et al, 1998) (Passechnik et al, 1998) (Krysinki et al, 1999).Supported BLMs can be used as molecular probes in biomedical analysis (Tien et al, 1998).

The close surface proximity of lipid bilayer membranes at the solid surface restricts or even prevents the incorporation of large transmembrane spanning proteins. The membrane – substrate distance is not sufficiently large to avoid direct contact between transmembrane proteins incorporated in the lipid bilayer membrane. Such membrane systems can’t be used for the detection of ion transport. Because embedded transmembrane proteins often have hydrophilic sections that protrude outside the lipid bilayer and may become immobile or denatured upon contact with the solid support. Moreover, its resistance is smaller and specific capacitance is 20-50 times

higher than that of conventional bilayer lipid membranes (Zviman and Tien, 1991) (Laptkova et al, 2005).

1.12 SALT BRIDGE SUPPORTED BILAYER LIPID MEMBRANES (sb-BLMs).

Salt bridge or agar gel supported bilayer lipid membrane system was developed to overcome the shortcomings of s-BLMs. By the application of sol-gel technology the bilayer lipid membrane is formed on the agar surface (Navratil et al, 2011). Introduction of gel electrode (Osakai et al, 1984) has stimulated progress in electroanalysis at liquid|liquid interfaces. The gel support is prepared by mixing agar with suitable electrolyte at elevated temperature and then cooled in a suitable mould to get gel electrode. This gel electrode is easy to handle and provides variety of shapes (Navratil et al, 2011).

Agar gel supported BLM can be accessed by a variety of surface analysis tools. In a number of reports it has been shown that EIS is useful as a nondestructive and sensitive method for investigation of lipid films on solid surfaces (Legin et al, 2007). The theoretical analysis of EIS data by fitting to a model represented by an equivalent electrical circuit allows the understanding of processes associated with the biomaterial-functionalized electrode surfaces (Legin et al, 2007).

1.13 POLYMER SUPPORTED BILAYER LIPID MEMBRANES (ps-BLMs)

To combine the most benefits of unsupported BLMs and solod supported BLMs, such as bilayer fluidity and stability, accessibility to various characterization methods, and the possibility of incorporation and investigation of membrane proteins the solid surfaces were separated from bilayer lipid membranes by a polymer cushion. This resulted in ps-BLM. These ps-BLMs are stable and the incorporation and characterization of proteins were made possible due to presence of a thin, lubricating water layer between the substrate and the inner monolayer. The ps-BLMs show lower impedance which is insufficient to match the electrical properties of biomembranes. Lower impedance of ps-BLMs limited their applications in biosensors.

1.14 BLM AS A MODEL FOR NERVE CELL MEMBRANE

Unlike other membranes, the nerve membrane contains the highest percentage of lipids and smallest percentage of proteins. For e.g. human myelin consists of about 80% lipids and only 20% proteins (Albert Lehningeret al, 1993) (Uma Maheswari et al, 2000). Therefore, nerve cell seems to be the exceptional structure that provides the best experimental support for the bilayer leaflet model. Each myelin layer encompasses two cell membranes of opposite orientation. The thickness of myelin by X-ray diffraction studies gives a solid proof for the layers present and the bilayer leaflet model of membrane.

1.15 STAGES IN THE FORMATION AND STABILIZATION OF BLM

When membrane forming lipids are placed in contact with aqueous medium, spontaneous thinning and stabilization of BLM results as it involves the formation of a structure with least free energy[27]. Initially, when lipid dispersion is applied to the membrane supporting aperture, it covers the aperture like a lens. Then due to spontaneous exclusion of solvent molecules and due to the Plateau-Gibbs border suction, self association of lipid molecules results, finally, in a bilayer[27, 29, 40, and 41]. The formation of membrane, once initiated, proceeds to a structure with minimum possible free energy.

The final thinning step has been attributed to the chance contact between the two leaflets of lipids. When the interfaces approach sufficiently closer together, van der Waals attractions developed between the hydrocarbon chains help in the ordering and stabilization of the bilayer structure. In forming this bilayer, adjacent molecules are drawn sufficiently close to be attached to the opposite interface. This effect, known as Zipper effect, is responsible for the growth of BLM[27, 28]. The various stages in the thinning of BLM are depicted schematically in Figure 1.8.

Membrane formation process is the first stage of any experiment on bilayer lipid membranes. The quality of membranes obtained decides about the usefulness of membrane for further study. The bilayer is always

surrounded by the Plateau-Gibbs border. Membranes with smaller border (higher bilayer area) are more stable and the phenomena in the border have lower influence on the observed results. When polarizing potentials are applied, the area of the bilayer increases as the P-G border is moved towards the periphery at the aperture. Thickness of the bilayer decreases due to electrocompression of the membrane at an external applied potential. Again, the extent of compression and increase in the area of BLMs depend on the nature of lipids, solvents used, concentration and nature of the ions present in the bath medium and so on. Not all the membranes formed are useful for the experiments, as evident from the photographs shown in Table 1.3.

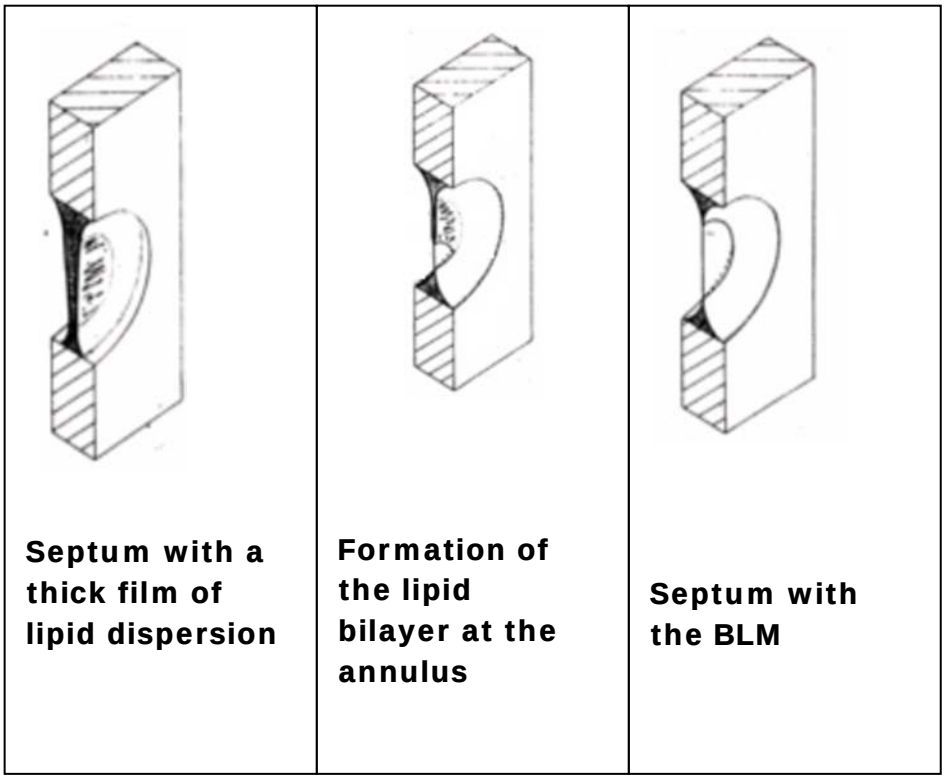
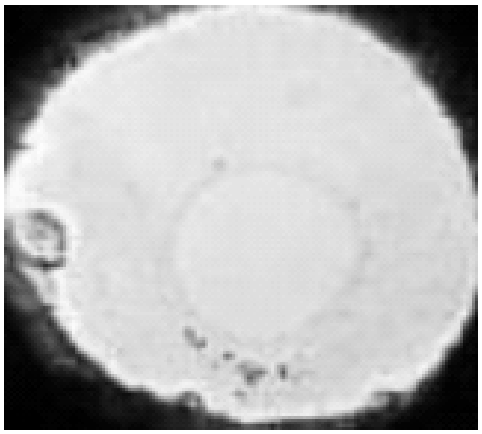
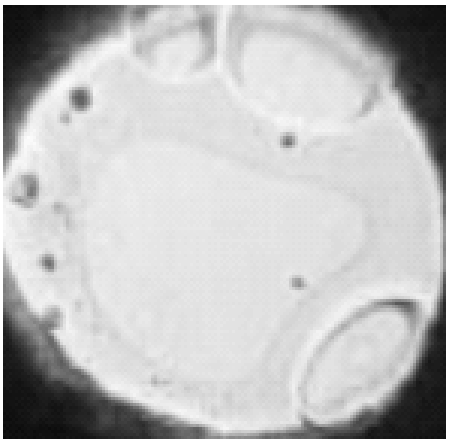
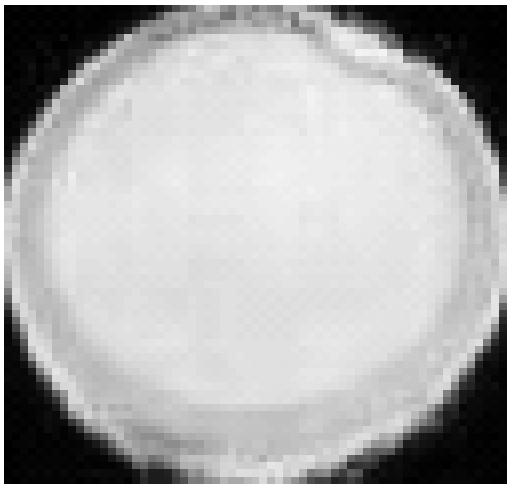
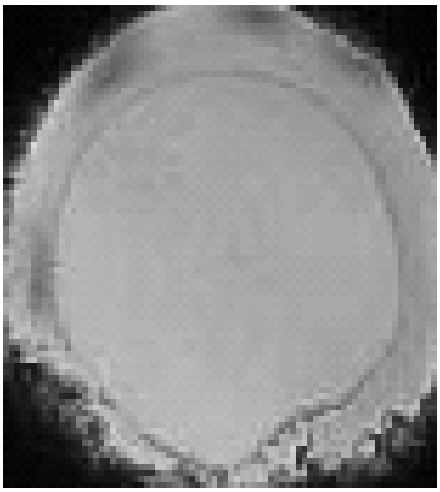


Figure 1.8 Various stages during thinning and stabilization of BLM.

Table 1.3

Photographs of bilayer lipid membranes

Membranes	**Observations**
	
The P-G border in this membrane is large and the bilayer occupies small part of the hole. This membrane is of little use for further experiments.	Border of the membrane contains bubbles of gas. This membrane is not useful for further experiments.
	
This membrane has a large bi-layer area and hence is stable and useful for further experiments.	The P-G border is moved to the periphery of the aperture under the influence of an applied polarization potential.
Slawomir Kalinowski, PhD, Home page: http://moskit.uwm.edu.pl/~kalinow/	

The true area of BLM is determined using optical measurements. The quality of a useful BLM can also be identified based on the capacitance or conductance reached after the stabilization of membrane. Since the area and other values vary from membrane to membrane, usually the experiments are repeated by a sufficient number of times in a given set of conditions and the mean average of the results is quoted. Usually, the absolute values of conductance, capacitance etc. of different membranes are not compared.

1.1 CENTRAL NERVOUS SYSTEM (CNS) DEPRESSANTS

1.16.1 Classification of CNS depressants

Several CNS depressants are used in clinical practice. The important classes of CNS depressants are

(1) Benzodiazepines e.g., diazepam (Valium), midazolam (Versed), clonazepam (Klonopin), lorazepam (Ativan) etc., (2) Barbiturates (Amobarbital), pentobarbital (Nembutal), thiopental (Pentothal) and (3) Miscellaneous agents such as paraldehyde (Paral), meprobamate (Miltown), ethchlorvynol (Placidyl) etc.

1.16.2 Effects of drugs on the central nervous system

Drugs may affect the functions of CNS in two ways.

1. Interaction with interneuronal signaling
    - Biosynthesis - enzymes and precursors
    - Storage

- Release (modulation by nerve terminal receptor)
- Inactivation (cellular uptake, enzymatic degradation)
- Receptor (signal binding sites, allostearic modulatory site)
- Post-receptor mechanisms (transduction, 2^{nd} and 3^{rd} messenger)

2. Interaction with ion-transport
    - Ion pumps
    - Ionophores
    - Fixed channels (K^+)
    - Voltage-operated channels (Na^+, K^+, Ca^{2+})
    - Receptor-operated channels (Na^+, K^+, Ca^{2+}, Cl^-)
    - Receptor modulated voltage-operated channels
    - Channels operated/modulated by intracellular messengers, e.g. ions, cyclic nulceotides, protein kinases

1.17 MECHANISM OF ACTION OF INTRAVENOUS ANESTHETICS

Barbiturates, benzodiazepines etc., bind to discrete receptor-sites on the chloride channel that is gated by the inhibitory neurotransmitter, γ -amino butyric acid (GABA). When intravenous anesthetics interact with GABA ionophore, GABA binding is increased and the channel spends more time in the open-form. This favors an increase in chloride ion conduction and a decrease in neuronal excitability. Though many mechanisms are possible, it is probably the main mechanism of action of intravenous anesthesia. An account

on the specific and non-specific interactions of benzodiazepines with the receptors and membranes will be taken up later.

1.18 BENZODIZAEPINES

Benzodiazepines are perhaps, the most widely prescribed drugs in the world. Anesthesiologists use benzodiazepines for a variety of purposes. Benzodiazepines are used as pre-medicant to reduce anxiety. They produce sedation and amnesia in conscious patients. They are also used in similar procedures that are performed under local or regional anesthesia. In large doses, they induce general anesthesia. Benzodiazepines are relatively unique in their ability to produce profound antirograde amnesia with unconsciousness. They also have marked anxiolytic effect.

Compared to other anesthetic agents, benzodiazepines are extraordinarily safe in many ways. They have high therapeutic indices (ratio of lethal to therapeutic dosage). However, when combined with other CNS depressants, benzodiazepines are found to be extremely hazardous.In particular, benzodiazepines are frequently combined with opioids to produce sedation or in large doses, to produce general anesthesia. The effects on breathing and cardiovascular systems are synergistic under these circumstances. Even relatively small doses of benzodiazepines and opioids in combination produce apnea. The three benzodiazepines most widely used (by intravenous) by anesthesiologists are diazepam, lorazepam, and midazolam.

1.18.1 History

Benzodiazepines were discovered accidentally. Chlordiazepoxide (Librium) was the first benzodiazepine synthesized by Sternbach in 1955 but he discarded the drug because it was presumed to be inert. In 1957, chlordiazepoxide was found to have hypnotic effect. Sternbach synthesized diazepam in 1956. The sedative and hypnotic effects of diazepam were discovered in 1957, and the drug was released for clinical practice in 1960. Diazepam was the first benzodiazepine to be used to produce anesthesia in 1966[3]. Bell synthesized oxazepam in 1961. The next major achievement was synthesis of the water-soluble midazolamby Fryer and Walser in 1976[4-6]. The existence of a benzodiazepine receptor (BZR) was first discussed in Milan in 1971. Its isolation and receptor-ligand interactions were demonstrated in 1977[7, 8] which have resulted in the generation of a number of new ligands and specific antagonists[9-11]. On March 18th, 1997, Roche Laboratories announced that it has received clearance for the continuous use of midazolam intravenously for sedation[12]. Previously, midazolam had been approved only for intermittent intravenous (IV) injection for sedation. Though more than 50 or so benzodiazepines have been developed, only four of them are available for intravenous use, namely, diazepam, lorazepam, midazolam and flunitrazepam.

1.18.2 Chemical structure of benzodiazepines

All benzodiazepines have the same basic characteristic structure and differ significantly from other classes of drugs. The term ***benzodiazepine*** refers to that part of the structure composed of a benzene ring (A), fused to a seven membered diazepine ring (B). The basic benzodiazepine nucleus is shown in Figure 1.9.

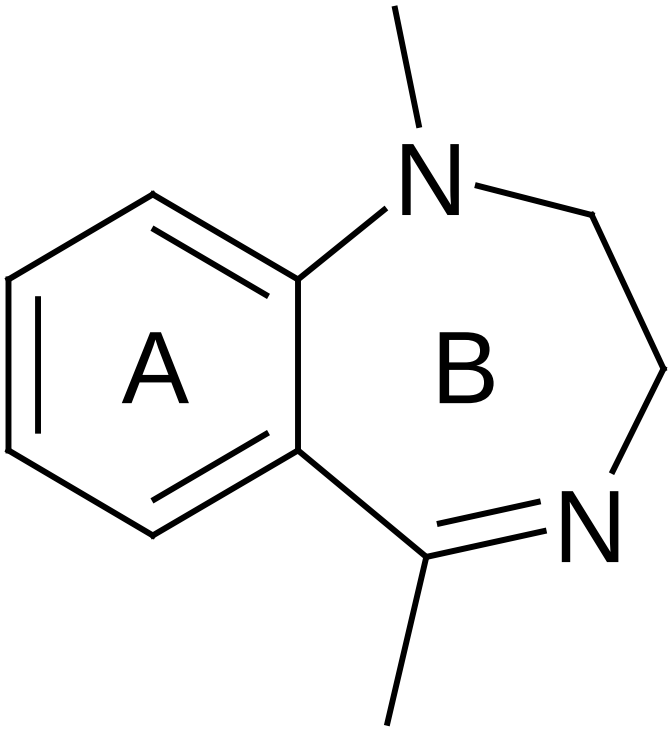


Figure 1.9 Basic benzodiazepine nucleus.

A number of commercially available benzodiazepines except alprazolam, estrazolam, midazolam, quazepam, triazolam and chlordiazepoxide have the same characteristic chemical structure but differ in substitutions at $R_{1,}$ $R_{3,}$ $R_{7,}$ $R_{2'}$ positions as shown in Figure 1.10.

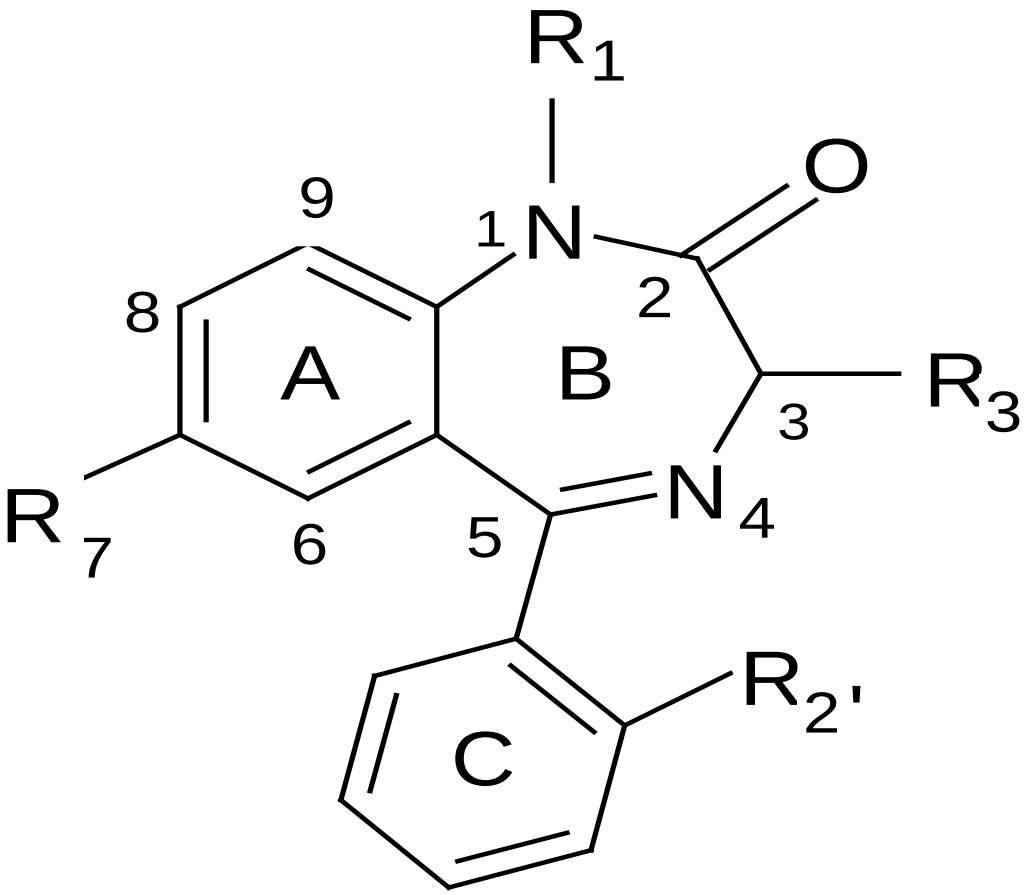


Figure 1.10 Basic benzodiazepine structure.

1.18.3 Lorazepam

Lorazepam is a short-acting benzodiazepine used in severe anxiety disorders and insomnia, in convulsions, as a premedicant and sedative for surgical and other procedures. Dependency may develop after regular use of the drug. Withdrawal symptoms may be particularly severe. Drowsiness, sedation, and ataxia are the most frequent adverse effects[13]. Lorazepam may be administered by oral, intravenous or intramuscular route. It is a very effective benzodiazepine but, it has a slow onset and relatively long duration of action that are often inconvenient in anesthesia practice. Often, it is rated as a short-acting drug; because it has no pharmacologically active metabolites (both diazepam and midazolam have active metabolites).The duration of action of lorazepam appears to be determined by receptor binding kinetics rather than plasma pharmacokinetics. This aspect is discussed in later sections.

1.19 DRUG-MEMBRANE INTERACTIONS: SIGNIFICANCE FOR MEDICINAL CHEMISTRY

The biological activity of number of drugs in pharmacology is known to directly depend on their interaction with biomembranes (Seydel and Wiese, 2002). The action of drugs on the surface of membrane as well as inside the cells is limited by the membranes. In the latter case also the drugs interacted with the membrane in order to cross it and reach the target (Lucio et al, 2010). The biological activity of the drug mainly depends on its efficiency to interact

with biomembranes (Lucio et al, 2010). The drugs which bind to proteins and regulate their activities bring the structural changes in the lipid phase resulting in structural defects in biomembranes, which in turn disturb membrane function and indirectly modulate membrane proteins (Lucio et al, 2010).

Experimental work on artificial membranes using a variety of methods revealed that the membrane properties may strongly be affected by the presence of membrane associated molecules. Evidence is also increasing that the presence of a heterogeneous lateral organization may have a number of important consequences on this interaction, including enhancement of penetration and insertion of the molecules at the domain boundary (Mouritsen and Jørgensen, 1998). Monte Carlo calculations on model membranes have also revealed that application of a drug may itself have an influence on the lateral organization of the lipid membrane (Mouritsen and Jørgensen, 1998). Accordingly, understanding the interaction of drugs with lipid membranes has long been a key issue in membrane biophysics and pharmaceutical research. Thus, studies on the clinical significance of drug-membrane interaction have gained a special focus and reinforce the importance in the field of medicinal chemistry since they constitute stimulating opportunities for understanding drugs mode of action and toxic effects which can’t be overlooked during drug synthesis and design.

1.19.1 Possibilities for Non-Specific Interaction of Drugs with membranes

Since the prime objective of this study is towards establishing non-specific interactions of benzodiazepines on membranes, the following relevant details are given in this section.

1.19.2 Lipid bilayer as the target

A nanometer scale molecule, after approaching the cell, will strike the part of the cell wall, which is in its reach and cannot search for a specific receptor site. If the place where it strikes the receptor is located in its required conformation, it will receive the molecule and either transport the molecule into the cell or will bring out other biochemical events and pharmacological actions. On the other hand, when the molecule strikes the bare membrane plane (where there is no receptor site), it will tend to partition and penetrate through the membrane into the cell depending on its lipid solubility. Thus, for any drug, though specific receptors are present, the possibility for site non-specific interaction cannot be excluded. The cell membrane is analogous to the protective wall around a house with proteins and receptors acting as doors and windows. The interior cell organelles are like valuables in the house. The entry of a drug through the receptors into the cell is a legitimate one through gates and doors of the house, while the entry through membrane is like entering into the house by breaking the wall[25].

Specific interaction of drugs are programmed and controlled by cells, whereas non-specific interactions are not. Since the bilayer construct is a common feature for all types of cell membranes, non-specific disturbances to membrane could occur to all cells including target cells. Such site-nonspecific interactions (Drug–Lipid interactions) are proposed to be the cause for undesirable side-effects such as neurotoxicities[26].

The transport of drugs into cells through specific receptors and carriers has been studied using in vitro and in vivo models. But, in order to study the interaction of drugs with lipid bilayers, model membranes which mimic the lipid bilayer architecture are very helpful[21, 27-29].

Thus, artificial membrane systems containing definite lipid compositions have been constructed and several individual events that occur in membrane were investigated. Use of artificial membranes gives us an insight into ion-transport mechanism operative in cell membranes and mitochondrial membranes.

1.19.3 Is drug action on receptors specific or non-specific?

The diversity of the structure of molecules which can act as CNS depressants indicates that there are no common receptors. The action of anesthetics is non-specific and physical. After the demonstration by Mayer and Overton that anesthetic potencies correlate to their solubility into olive oil, the lipid solubility theory monopolized the anesthesia theories for almost a

century. Later, the specific receptor theories, which stressed on receptor bindings, became the top mode. Nevertheless, a large number of different kinds of anesthetic molecules and wide variety of responding systems are difficult to reconcile with specific interaction concept [1].

Anesthetics are unique drugs in pharmacology. They affect all the macromolecules. The only comparable drugs are disinfectants. Both anesthetics and disinfectants are non-specific drugs. The lipid theories are often misinterpreted that anesthetic action site is the lipid membranes. There is a major difference between solvation into unstructured olive oil and binding to structured lipid membranes. There are no units for hydrophobicity. When the dielectric constant is used for hydrophobicity, olive oil is about 10, water is 80, and the core of lipid bilayer is 1.8[30]. Nevertheless, the correlation that covers 5 orders of magnitude is just amazing[1].

After the establishment of Fluid-Mosaic model of lipid membranes by Singer and Nicolson, the membrane fluidity theory became the fad in membrane biology and the protein theory became the top fashion. The article presented at New York Academy of Science Symposium[31] proposed that a vast majority of the receptors like nicotinic acetylcholine receptor, $GABA_A$ receptor, $GABA_B$ receptor, NMDA receptor, non-NMDA glutamate receptors, glycine receptor, peptide receptor, G-protein linked systems, alpha 2-adrenergic receptor, Na^+ channel, K^+ channel and chloride ion channel are all affected by volatile anesthetics. In addition, volatile anesthetics affect the pre-

synaptic transmitter release. A number of enzymes are also affected by volatile anesthetics. This means that, almost all the channels are affected when the concentration of anesthetics is increased. Hence, Ueda refers these anesthetic receptor interactions also as non-specific[1].

Channels are highly organized lipoproteins. It is impossible to isolate active channel proteins in lipid-free form and to distinguish anesthetic interaction with proteins from the supporting lipid bilayer. In modern terminology, anesthesia should result from a reversible state of equilibrium where the channel and receptor proteins undergo reversible conformational changes[1].

Biophysical studies attempt to find physicochemical mechanisms that dictate the interaction of anesthetic molecules with proteins and lipids. Ueda states that there is no system that is uninfluenced by anesthetics. Ueda and Suzuki[32,33] demonstrated that anesthetics unfolded and expanded firefly Luciferase, whereas 14-carbon fatty acid (myristate) tightened the structure. Myristate has no action on channels, proposed to be the targets for anesthetic action. In this context, it is futile to pinpoint one neurotransmitter system for the anesthetic target site. Large numbers of neurotransmitter systems respond to anesthetics with high cooperativeness. There is no single button that can be pushed to induce anesthesia. This means that even when the receptor bindings are responsible for CNS activities, all possible receptors are affected. Actually, this is again a non-specific action.

1.19.4 Lipid/Protein interface as the target

As described earlier, there has been a long-standing controversy on whether membrane lipids or proteins are the targets for general anesthetics. The lipid/protein interface is also a possible target site because many membrane enzymes and gated-channel proteins such as nicotinic acetylcholine receptors require lipids for activity. Doris P. Flugmacher et al. have reported that the lipid/protein interface rather than protein or lipid alone appeared to be the anesthetic target site[34].

1.20 CHARACTERIZATION OF DRUG-MEMBRANE INTERACTIONS

Many sophisticated techniques have been developed and used by different workers to characterize drug-membrane interactions. Techniques such as phase transition studies[42-44], spectrofluorimetric analysis[45-48], NMR[49], FTIR spectroscopy[39], Differential Scanning Calorimetry[50-52]etc., have been found to be useful in studying drug-membrane interactions. Based on the results of such techniques, the drug-induced changes in the microscopic structure of the membranes have been worked out.

Since electrical measurements provide simple means to characterize drug-membrane interactions, they have been used extensively by various workers[53-58]. The instruments required for electrical measurements are reliable and economical. The simple electrical equivalent circuit of a BLM is a RC

couple i.e. a capacitor and a resistor in parallel. Figure 1.11 gives simple electrical equivalent circuit of BLM. Many electrical parameters of the membranes such as AC capacitance, impedance, resistance, DC conductance and capacitance etc. are followed easily using suitable instrumentation. The AC measurements give an insight into the mechanism of drug-membrane interaction. It is possible to distinguish between surface and interior interactions of drugs with the membrane by following changes in the AC electrical parameters[59]. The DC studies provide an insight into the changes produced in the permeability characteristics of BLMs[27].

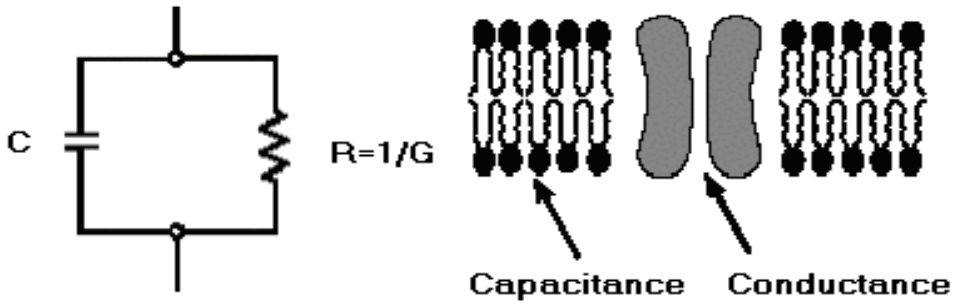


Figure 1.11 Membrane Behavior compared to electrical circuit.

The experimental parameters to study these interactions include variations in temperature, pH, concentration and nature of the bath medium apart from the drug concentrations. In the present study, both AC and DC measurements were employed to characterize drug-membrane interactions.