

Physical Properties and Functional Roles of Lipids in Membranes

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INTRODUCTION
AND OVERVIEW

Biological membranes contain an astonishing variety of lipids. As detailed throughout this book, generation of this diversity requires elaborate metabolic pathways. The lipid compounds representing the end products of these pathways must bestow significant evolutionary advantages to the cellular or multicellular systems in which they reside, implying particular functional roles for each component. However, clarification of the functional roles of individual lipid species has proven a difficult problem. Here we present a synopsis of the physical properties of lipid systems and indicate how they may relate to the functional capacities of biological membranes.

The major role of membrane lipids has been understood in broad outline since the early experiments of Gorter and Grendell (1925), who extracted lipids from the erythrocyte membrane and measured the area these lipids were able to cover as a monolayer at an air-water interface. Although a number of unwarranted assumptions were made in the analysis of these data, the errors fortunately compensated for one another and led to the correct conclusion that the erythrocytes contained sufficient lipid to provide a bilayer lipid matrix surrounding the red blood cell. This bilayer lipid organization, which provides a permeability barrier between exterior and interior compartments, was further characterized by Danielli and Davson (1935) and has remained a dominant theme in our understanding of the organization and function of biological membranes. Subsequent observations that such bilayers are fluid, allowing rapid lateral diffusion of lipid and protein in the plane of the membrane, and that

membrane proteins are often inserted into and through the lipid matrix have further contributed to our present understanding of membranes, resulting in the Singer and Nicolson (1972) *fluid mosaic model*, a refined version of which is shown in Figure 2.1.

The ability of lipids to assume the basic **bilayer** organization is dictated by a unifying characteristic of membrane lipids—namely, their *amphipathic* character, which is indicated by the presence of a

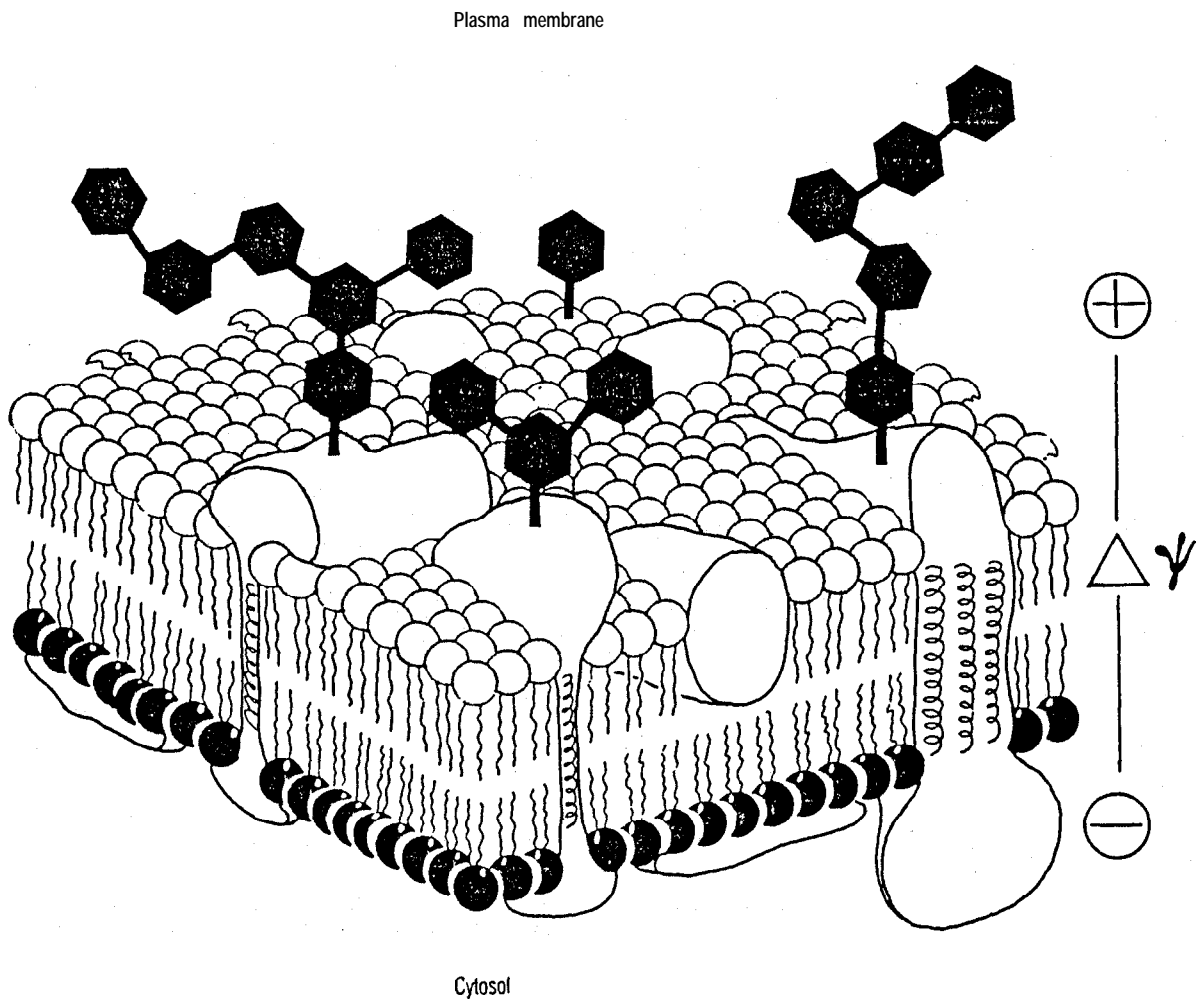


Figure 2.1. The topography of membrane protein, lipid, and carbohydrate in the fluid mosaic model of a typical eucaryotic plasma membrane. Phospholipid asymmetry results in the preferential location of phosphatidylethanolamine and phosphatidylserine in the cytosolic monolayer. Carbohydrate moieties on lipids and proteins face the extracellular space. $\Delta\psi$ represents the transmembrane potential, negative inside the cell.

polar or hydrophilic (water loving) head group region and nonpolar or hydrophobic (water hating) region. The chemical nature of these hydrophilic and hydrophobic sections can vary substantially. However, the lowest-energy macromolecular organizations assumed in the presence of water have similar characteristics, where the polar regions tend to orient toward the aqueous phase, while the hydrophobic sections are sequestered from water. In addition to the familiar bilayer phase, a number of other macromolecular structures are compatible with these constraints, as indicated later in this chapter. It is of particular interest that many naturally occurring lipids prefer nonbilayer structures in isolation.

The fluidity of membranes depends on the nature of the acyl chain region comprising the hydrophobic domain of most membrane lipids. Most lipid species in isolation can undergo a transition from a very viscous gel (frozen) state to the fluid (melted) *liquid-crystalline* state as the temperature is increased. This transition has been studied intensively, since the local fluidity, as dictated by the gel or liquid-crystalline nature of membrane lipids, may regulate membrane-mediated processes. However, at physiological temperatures most, and often all, membrane lipids are fluid; thus, the major emphasis of this chapter will concern the properties of liquid-crystalline lipid systems. As indicated later, the melted nature of the acyl chains depends on the presence of cis double bonds, which can dramatically lower the transition temperature from the gel to the liquid-crystalline state for a given lipid species.

The ability of lipids to self-assemble into fluid bilayer structures is consistent with two major roles in membranes: establishing a permeability barrier and providing a matrix with which membrane proteins are associated. Roles of individual lipid components may therefore relate to establishing appropriate permeability characteristics, satisfying insertion and packing requirements in the region of integral proteins (which penetrate into or through the bilayer), as well as allowing the surface association of peripheral proteins via electrostatic interactions. All these demands are clearly critical. An intact permeability barrier to small ions such as Na^+ , K^+ , and H^+ , for example, is vital for establishing the electrochemical gradients which give rise to a membrane potential and drive other membrane-mediated transport processes. In addition, the lipid in the region of membrane protein must seal the protein into the bilayer so that nonspecific leakage is prevented and an environment appropriate to a functional protein conformation is provided.

In summary, membrane lipids satisfy demands related to membrane structure, fluidity, and permeability, as well as protein association and function. These aspects will be dealt with at length; however, before a coherent discussion is possible, a basic overview of

lipid diversity in membranes; a study of the methods employed to isolate individual components, and a discussion of the physical properties of lipids are essential. These will comprise the bulk of the next three sections.

LIPID DIVERSITY AND DISTRIBUTION

The general definition of a *lipid* is a biological material soluble in organic solvents, such as ether or chloroform. Here we shall discuss the diverse chemistry of the subclass of lipids which are found in membranes. This excludes other lipids which are poorly soluble in bilayer membrane systems, such as fats (triglycerides) and cholesterol esters.

Chemical Diversity of Lipids

The major classes of lipids found in biological membranes are summarized in Figure 2.2. We shall discuss most of these compounds in depth at various points in this book; we present only a brief synopsis here. In eucaryotic membranes the glycerol-based phospholipids are predominant, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and **cardiolipin**. Sphingosine-based lipids, including sphingolipids and the **glycosphingolipids**, also constitute a major fraction. The glycolipids, which can also include carbohydrate-containing glycerol-based lipids (found particularly in plants), play major roles as **cell-surface-associated antigens** and recognition factors in eucaryotes. The physical properties of glycolipids have not been extensively characterized and will not be discussed in this chapter. Cholesterol is also a major component of eucaryotic membranes, particularly in mammalian plasma membranes, where it may be present in equimolar proportions with phospholipid.

In most procaryotic membranes, phosphatidylcholine is not usually present; the major phospholipids observed are **phosphatidylethanolamine**, phosphatidylglycerol, and cardiolipin. In plant membranes on the other hand, lipids such as monogalactosyl and digalactosyl diglycerides can form the majority components of membranes such as the chloroplast membrane.

These observations give some impression of the lipid diversity in membranes, but it must be emphasized that this diversity is much more complex. Minority species such as sulfolipids, phospholipids with phosphorylated head groups, and lysolipids abound. Furthermore, each lipid species exhibits a characteristic fatty acid composition. In the case of glycerol-based phospholipids, for example, it is usual to find a saturated fatty acid esterified at the 1-position of the glycerol backbone and an unsaturated fatty acid at the **2-position**. Also, in eucaryotic membranes it is usual to find that phosphatidyl-

Table 2.1. Gas Chromatographic Analyses of the Fatty Acid Chains in Human Red Cell Phospholipid

Chain length and unsaturation	Total phospholipids	Sphingomyelin	Phosphatidylcholine (lecithin)	Phosphatidylethanolamine	Phosphatidylserine
16:0*	20.1	23.6	31.2	12.9	2.7
18:0	17.0	5.7	11.8	11.5	37.5
18:1	13.3	+	18.9	18.1	8.1
18:2	8.6	+	22.8	7.1	3.1
20:0	+ [†]	1.9	+	+	+
20:3	1.3	—	1.9	1.5	2.6
22:0	1.9	9.5	1.9	1.5	2.6
20:4	12.6	1.4	6.7	23.7	24.2
23:0	+	2.0	+	+	+
24:0	4.7	22.8	+	+	+
22:4	3.1	—	+	7.5	4.0
24:1	4.8	24.0	+	+	+
22:5	2.0	—	+	4.3	3.4
22:6	4.2	—	2.1	8.2	10.1

Note: The data are expressed as weight % of the total.

* This code indicates the number of carbon atoms in the chain and the number of double bonds.

[†] Denotes that the concentration did not exceed 1% of the total.

Reproduced with permission of van Deenen and de Gier (1974).

ethanolamine and phosphatidylserine, for example, are more unsaturated than other phospholipids. In order to give a true impression of the molecular diversity of phospholipids in a single membrane, we list in Table 2.1 the fatty acid composition of phospholipids found in the human erythrocyte membrane. From this table and other analyses (van Deenen and de Gier 1974) it is clear that the number of different molecular species of phospholipids in a membrane can easily exceed 100.

The lipid composition of membranes can vary dramatically among different cells or organelles. In addition, different sides or monolayers of the same membrane can contain different lipid species. These different compositions are indicated in the following sections.

Membrane Lipid Compositions

The lipid compositions of several mammalian membrane systems are given in Table 2.2. Dramatic differences are observed for the cholesterol contents: Plasma membranes such as those of myelin or the erythrocyte contain equimolar quantities of cholesterol and phospholipid, whereas the organelle membranes of endoplasmic reticulum or the inner mitochondrial membrane contain little or no cholesterol. This cholesterol distribution correlates well with the distribution of

Table 2.2. The Lipid Composition of Various Biological Membranes

Lipid	Erythrocyte*	Myelin •	Mitochondria† (inner and outer membrane)	Endoplasmic reticulum†
Cholesterol	23	22	3	6
Phosphatidylethanolamine	18	15	35	17
Phosphatidylcholine	17	10	39	40
Sphingomyelin	18	8	—	5
Phosphatidylserine	7	9	2	5
Cardiolipin	—	—	21	—
Glycolipid	3	28	—	—
Others	13	8	—	27

Note: The data are expressed as weight % of total lipid.

* Human sources.

† Ret liver.

sphingomyelin. Cholesterol may have a “fluidizing” role in membranes containing sphingomyelin, which is relatively saturated.

Cardiolipin is almost exclusively localized to the inner mitochondrial membrane, and it has been suggested that cardiolipin is required for the activity of cytochrome c oxidase, the terminal member of the respiratory electron-transfer chain. In general, the lipids of more metabolically active membranes are considerably more unsaturated, as indicated in Table 2.3.

It is interesting to note that the lipid composition of the same membrane system in different species can also vary significantly. The rat erythrocyte membrane, for example, contains low levels of sphingomyelin and elevated levels of phosphatidylcholine with respect to the human erythrocyte. In the bovine erythrocyte, this distribution is reversed, with high sphingomyelin, and low phosphatidylcholine, contents.

Table 2.3. Double-Bond Composition of Phospholipids of Various Membranes

Membrane	Number of double bonds per acyl chain
Myelin	0.5
Erythrocyte	1.0
Sarcoplasmic reticulum	1.4
Mitochondria (inner)	1.5
Nerve synapse	>2

Transbilayer Lipid Asymmetry

A major discovery of recent years has been the observation that the inner and outer leaflets of membrane **bilayers** may exhibit different lipid compositions (Op den Kamp 1979). Several different species of membranes have been investigated with respect to lipid asymmetry; however, the plasma membrane of human erythrocytes has been the most thoroughly investigated.

The results obtained indicate that most membranes display some degree of lipid asymmetry. The use of impermeable probes that react with the primary **amines** of phosphatidylethanolamine and **phosphatidylserine** on only one side of the membrane has shown that the majority of the amino-containing phospholipids of the erythrocyte are located on the inner monolayer. Combinations of chemical probes and phospholipase treatments indicate that in a normal red blood cell all the phosphatidylserine is located in the inner monolayer, whereas approximately 20% of the phosphatidylethanolamine can be detected at the outer surface, with 80% confined to the inner monolayer. The outer monolayer consists predominantly of phosphatidylcholine, sphingomyelin, and glycolipids. Figure 2.3 summarizes the **transbilayer** lipid distributions obtained for various mammalian cell membranes and viral membranes derived from animal-cell plasma membranes. A common feature is that the amino-containing **phospholipids** are chiefly limited to the cytosolic side of plasma membranes. It is interesting that the little information available for organelle membranes suggests that phosphatidylethanolamine and phosphatidylserine are also oriented toward the cytosol.

A general feature of plasma membrane asymmetry is that the majority of phospholipids that exhibit a net negative charge at physiological **pH** (phosphatidylserine and **phosphatidylinositol**—phosphatidylethanolamine is only weakly anionic) are limited to the cytosolic half of the bilayer. Certain proteins appear to be involved in maintaining this asymmetry. Treatment of erythrocytes with diamide, which induces cross-linking of the cytoskeletal protein **spectrin**, results in the appearance of phosphatidylserine in the outer monolayer. Pathological red blood cells known to have lesions associated with cytoskeletal proteins also exhibit a partial breakdown of asymmetry, with an increased exposure of phosphatidylserine and phosphatidylethanolamine on the outer half of the bilayer and an equivalent transfer of phosphatidylcholine to the inner monolayer (Lubin and Chiu 1982).

These experiments suggest a possible interaction between **cytoskeletal** proteins and membrane phospholipids. The functional importance of lipid asymmetry is not clear but could be related to prevention of exposure of phosphatidylserine at the outer surface of a normal cell (which has been suggested to be a signal of senescence (Tanaka and Schroit 1983)). Alternatively, phosphatidylserine may

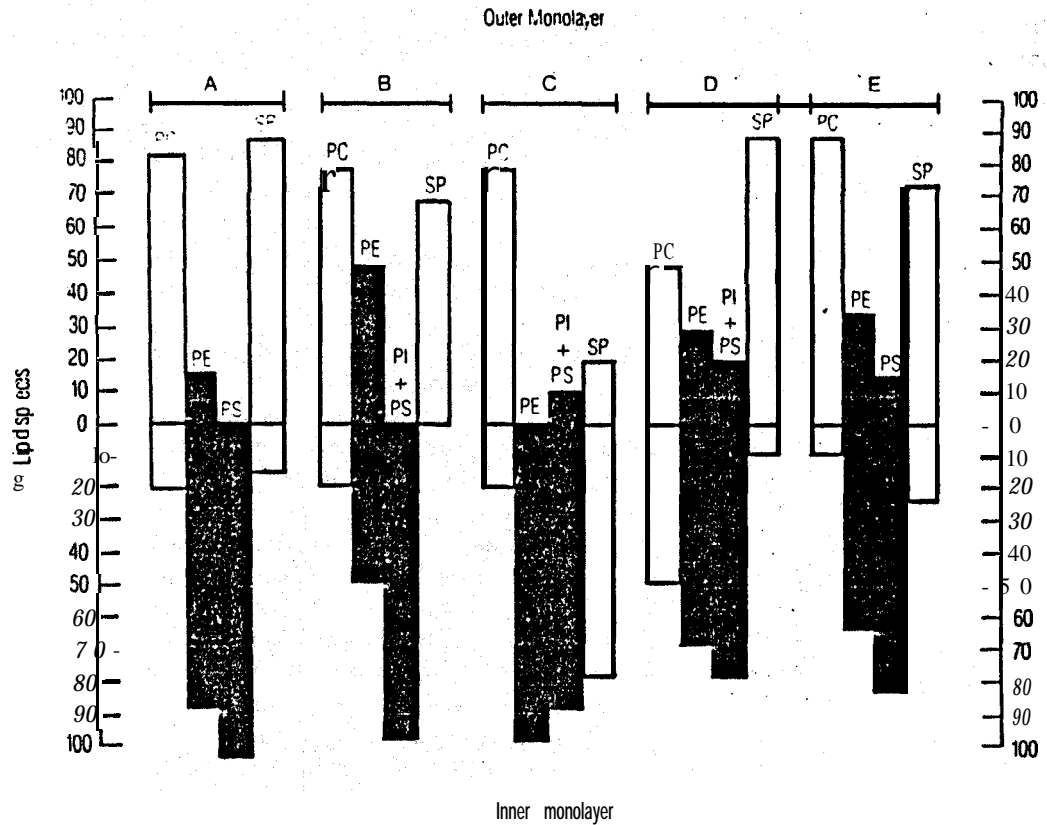


Figure 2.3. Phospholipid asymmetry in plasma membranes. (A) Human erythrocyte membrane, (B) rat liver blood sinusoidal plasma membrane, (C) rat liver continuous plasma membrane, (D) pig platelet plasma membrane, (E) VSV envelope derived from hamster kidney BHK-21 cells. The data were adapted from Houslay and Stanley (1982). See table 2.6 for phospholipid nomenclature.

be required at the cytosolic surface to maintain a functioning cytoskeleton.

MODEL MEMBRANE SYSTEMS

The physical properties and functional roles of individual lipid species in membranes are exceedingly difficult to ascertain in an intact biological membrane due to the complex lipid composition. In order to gain insight into the roles of individual components, it is necessary to construct *model membrane* systems that contain the lipid species of interest. This requires three steps, namely, isolation **or** chemical synthesis of a given lipid, construction of an appropriate model system containing that lipid, and subsequent incorporation of a particular protein if understanding the influence of a particular lipid on protein function is desired. By this method specific models of

biological membranes can be achieved in which the properties of individual lipid components can be well characterized.

Lipid Isolation and Purification

A variety of techniques has been developed for isolation of lipids from membranes (Kates 1973). These differ according to the particular source and type of lipid being isolated. A procedure commonly employed for the preparation of erythrocyte phospholipids is illustrated in Figure 2.4. A first step common to most procedures is to disrupt the membrane in a solvent system which denatures and precipitates most of the protein and **solubilizes** the lipid component. The Bligh and Dyer procedure is perhaps most often employed and involves incubation of the membrane system in a **chloroform-methanol-water (1:2:0.8)(v:v:v)** mixture, which forms a one-phase system. The subsequent addition of chloroform and water to the mixture containing the extracted lipids results in a two-phase system where the lower (chloroform) phase contains most membrane lipids.

Column *chromatography* is usually subsequently employed for isolation of individual lipid species. A solid phase such as silicic acid, DEAE cellulose, aluminum oxide, or carboxymethyl cellulose is used, depending upon the lipid being isolated, and lipids are eluted using mixtures of solvents with different polarities, such as chloroform and methanol. Thin-layer *chromatography* is generally used for lipid identification and for ascertaining purity. All these separation techniques rely upon the different partitioning characteristics of lipids between the stationary phase surface and mobile solvent phase for different solvent polarities. The exact nature of the binding of lipid to the solid phase is not well understood but appears to involve both electrostatic and hydrophobic interactions. Carboxymethyl cellulose and DEAE cellulose are often used for separation of anionic lipids.

Modern developments include *high-pressure preparative liquid chromatography*, which enables the rapid purification of large quantities of natural lipids (Pate1 and Sparrow 1978). Techniques for separating phospholipids according to the degree of acyl chain unsaturation are, as yet, tedious and expensive and normally utilize the tendency of unsaturated lipids to form complexes with certain metals; silver ions are commonly employed. Silica gel impregnated with silver nitrate can be used to prepare appropriate columns or thin-layer plates.

Reversed-phase chromatography (Skipski and Barclay 1969), where the stationary phase is hydrophobic and the mobile phase hydrophilic, is becoming more popular for separation of membrane lipids. The solid support is usually coated with hydrocarbon chains of a defined length (and consequently of regulated hydrophobicity), and the mobile phase is hydrophilic. This technique is particularly useful for **separating** single lipid classes according to their acyl chain length.

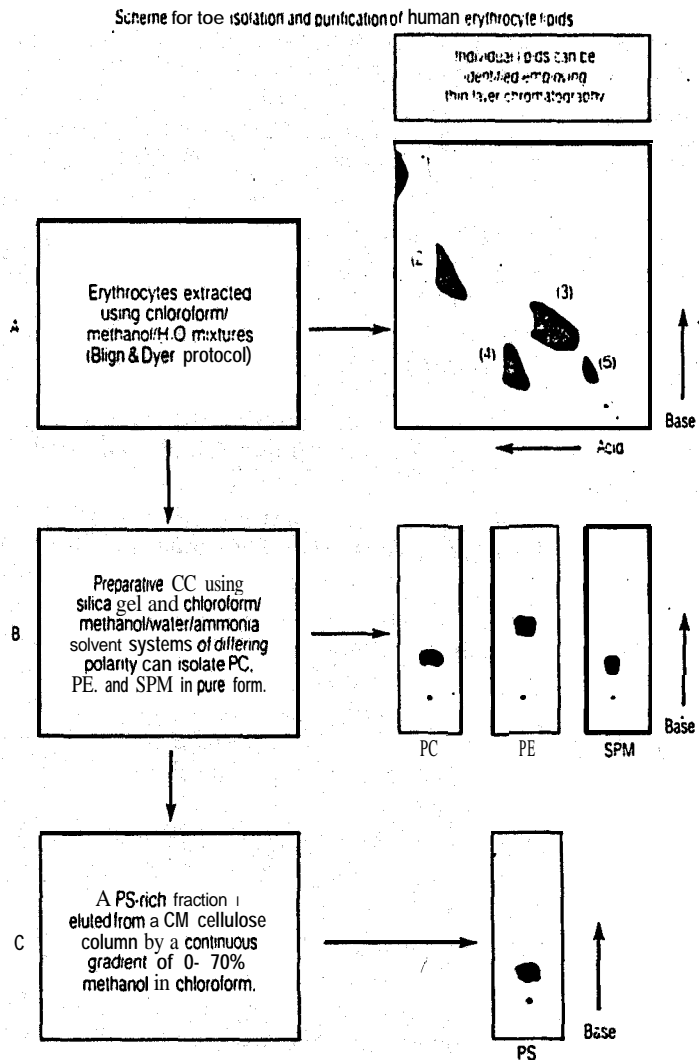


Figure 2.1. An outline of the procedure for extracting and purifying the phospholipid species of human **erythrocytes**. In step A, red cells are extracted using the Bligh and Dyer protocol. Denatured hemoglobin precipitates at this stage and is readily removed by centrifugation. Two-dimensional thin-layer chromatography is used to identify all the phospholipid species in the total lipid extract: (1) cholesterol, (2) PE, (3) PC, (4) PS, and (5) SPM. Step B utilizes preparative liquid chromatography (LC) to obtain pure PC, PE, and SPM. The PC and SPM fractions are readily separated using **chloroform/methanol/water (60:30:4, v/v)** to elute the lipid from the silica gel column. PE is further purified by passing the lipid once more through the column using **chloroform/methanol/water/25% ammonium hydroxide (60:30:1:1, v/v)**. In step C an impure PS fraction, obtained from the passes outlined above, is purified by elution from **carboxymethyl (CM) cellulose** using a continuous gradient of 0 to 70% methanol in chloroform.

For phospholipid nomenclature, see Table 2.6. Acid refers to the thin-layer plate running solvent **chloroform/methanol/acetic acid/water (60:30:8:3, v/v)** and base, to **chloroform/methanol/25% ammonium hydroxide/water (90:54:6:5, v/v)**.

Techniques for Making
Model Membrane
Vesicles

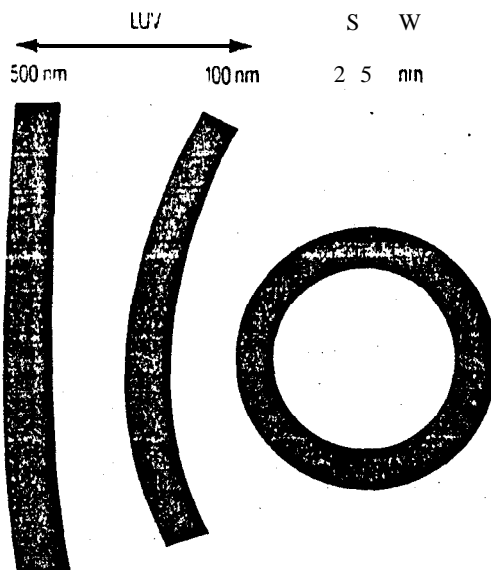
Once lipids have been isolated, purified, and chemically characterized, their properties as membrane components can be studied. For this purpose a number of techniques have been developed for producing model membranes from lipids. Preparation of the simplest model system involves the straightforward hydration of a lipid film by mechanical agitation, such as vortex mixing. *In the case of bilayer-forming lipids, this hydration results in a macromolecular structure which is composed of a series of concentric bilayers interspersed by narrow aqueous spaces (Bangham, Standish, and Watkins 1965). Such structures are usually referred to as liposomes or multilamellar vesicles (MLVs) and have been used for many years as models for the bilayer matrix of biological membranes. Their use is mostly restricted to physical studies on bilayer organization and the motionai properties of individual lipids within a membrane structure. MLVs are not ideal models for the study of other aspects of lipids in membrane structure and function, mainly because as little as 10% of the total lipid of a MLV is contained in the outermost bilayer. As a result, methods have been sought by which unilamellar (single bilayer) model membranes can be obtained either directly or from MLVs.*

Small unilamellar vesicles (SUVs) can be made from MLVs by subjecting the MLVs to ultrasonic irradiation (Huang 1969) or by passage through a French press (Barenholtz, Amselem, and Lichtenberg 1979). However, their small size limits their use in model membrane studies. Typically, diameters in the range 25-40 nm are observed. The radius of curvature experienced by the bilayer in SUVs is so small (Figure 2.5) that the ratio of lipid in the outer monolayer to lipid in the inner monolayer can be as large as 2:1. As a result of this curvature, the packing constraints experienced by the lipids perturb their physical properties in comparison with less highly curved systems. This restricts the use of SUVs for physical studies on the properties of membrane lipid. Moreover, the aqueous volume enclosed by the SUV membrane is often too small to allow studies of permeability or ion distributions between the internal and external aqueous compartments.

A more useful membrane model is the *large unilamellar vesicle (LUV)* system, where the mean diameter is larger, and the distribution of lipid between the outer and inner monolayers is closer to 1:1. The most common procedures for producing LUVs (outlined in Table 2.4) result in unilamellar vesicles with diameters ranging from 50 to 500 nm. These preparative procedures usually include the use of detergents (Mimms et al. 1981) or organic solvents (Szoka and Papahadjopoulos 1980), although LUVs can be produced directly from MLVs (Hope et al. 1985).

Procedures that employ detergents vary depending upon the type of detergent; however, the principle is the same. Lipids are solubi-

Curvature and some characteristics of large unilamellar and small unilamellar vesicles



Diameter (nm)	IM/OM (mole ratio)	Trap ($\mu\text{l per } \mu\text{mol}$)	No phospholipid molecules per vesicle	No. vesicles per μmol of lipid
25	0.36	0.2	3.6×10^7	1.7×10^{14}
100	0.81	2.7	8.0×10^4	7.6×10^{13}
500	0.96	17	2.2×10^6	2.7×10^{11}

Figure 2.5. **The curvature and some characteristics of large unilamellar vesicles (LUV) and small unilamellar vesicles (SW). LUVs typically have diameters in the range 100-400 nm. SUVs prepared by sonication can be as small as 25 nm in diameter. The radius of curvature for each vesicle size is shown in proportion. The ratio of lipid in the inner monolayer (IM) compared with lipid in the outer monolayer (OM) gives an indication of the packing restrictions in bilayers with a small radius of curvature. The trapped volume refers to the volume of aqueous medium enclosed per micromole of phospholipid. The calculations were made assuming a bilayer thickness of 4 nm and a surface area per phospholipid molecule of 0.6 nm^2 .**

lized by the detergent of choice (such as cholate or octylglucoside); then the detergent is removed either rapidly by dilution or gel filtration, or slowly by dialysis. As the detergent concentration decreases, the lipids adopt unilamellar vesicular structures. The

Table 2.4. Common Procedures for the Generation of Large Unilamellar Vesicles

<i>Category</i>	<i>Technique</i>	<i>Trapped Volumes</i> ($\mu\text{L}/\mu\text{mol lipid}$)	<i>Advantages</i>	<i>Disadvantages</i>
Detergent dialysis	Cholate dialysis	0.5-5	Large trapped volumes.	Detergents difficult to remove completely; procedures lengthy; low trapping efficiency; limited to certain lipid mixtures.
	Octylglucoside dialysis	~10		
Hydration from organic solvent	Reverse-phase evaporation	-10	High trapping efficiency for reverse-phase technique only; large trapped volumes.	Technically complex; limited to certain lipid mixtures; vaporization and injection techniques have low trapping efficiency; residual organic solvent.
	Ether vaporization	-10		
	Ethanol injection	0.5-5		
Direct from MLVs	MLVs extruded through 0.1- μm polycarbonate filters	1-2	High trapping efficiency for extrusion techniques only; no detergents or solvents used; fast procedures.	Trapped volumes low unless freeze-thaw protocol is employed.
	Plus freeze-thaw	1-10		
	Sonication	0.2-0.5		
	Plus freeze-thaw	1-10		

vesicle size can be controlled to some extent by the rate at which detergent is removed.

A number of methods exist for preparation of LUVs employing organic solvents (Szoka and Papahadjopoulos 1980). The lipid is first solubilized in an organic solvent which is subsequently diluted by aqueous buffer. The largest unilamellar vesicles are produced by injection procedures whereby lipid is dissolved in ether or ethanol, then slowly injected into aqueous buffer. An alternative protocol employing organic solvent is called the *reverse phase evaporation* procedure, which involves making an emulsion of lipid (dissolved in ether or mixtures of other organic solvents) and aqueous buffer. The organic solvent is carefully removed under partial vacuum, which gives rise to hydrated lipid in the form of a thick gel. This gel can be

diluted and sized by extrusion through polycarbonate filters of defined pore sizes to give LUVs. Finally, it is possible to form LUVs by repeated extrusion of MLVs through polycarbonate filters with pore sizes of 100 nm or less (Hope et al. 1985). An advantage of this procedure is that it does not require detergents or solvents, which are difficult to remove completely.

Techniques for Making Planar Bilayers and Monolayers

Planar bilayers (also known as *black lipid membranes*) are favorite model membranes of electrophysiologists interested in current flow across a bilayer (Fettiplace et al. 1974). They are formed by dissolving phospholipids in a hydrocarbon solvent and painting them across a small aperture (approximately 2 mm in diameter) which separates two aqueous compartments. The solvent tends to collect at the perimeter of the aperture, leaving a bilayer film across the center. The electrical properties of the barrier are readily measured employing electrodes in the two buffered compartments. It is also possible to incorporate some membrane proteins into the film, if the protein can be solubilized by the hydrocarbon. With this technique, ion channels have been reconstituted and voltage-dependent ion fluxes recorded. The most serious problem of black lipid membranes is the presence of the hydrocarbon solvent, which may change the normal properties of the lipid bilayer being studied.

With regard to monolayer systems, amphipathic lipids orient at an air-water interface. The result is a monolayer film which, in the case of phospholipids, represents half of a bilayer, where the polar regions are in the aqueous phase and the acyl chains extend above the buffer surface. Such films can be compressed and their resistance to compression measured. The study of compression pressure versus surface area (occupied by the film) yields information on molecular packing of lipids and lipid-protein interactions. Perhaps the best-known result of monolayer studies is the *condensation effect* of cholesterol and phospholipid, in which the area occupied by a typical membrane phospholipid molecule and a cholesterol molecule in a monolayer is less than the sum of their molecular areas in isolation. This phenomenon provides a strong indication of a specific interaction between this sterol and membrane phospholipids (Demel and de Kruijff 1976).

Reconstitution of Integral Membrane Protein into Vesicles

An important step, both for the study of membrane protein function and for the building of simple but more representative biological membranes, is the insertion of purified integral membrane proteins into well-defined lipid model membranes. A large variety of

membrane proteins have been reconstituted (Racker 1973). For the purpose of discussing the salient features of reconstitution techniques, we shall use the example of cytochrome *c* oxidase from bovine heart mitochondria. This integral membrane protein, which has been purified and is relatively well characterized, spans the inner mitochondrial membrane and oxidizes cytochrome *c* in the terminal reaction of the electron-transfer chain.

Purified integral proteins such as cytochrome oxidase maintain a functional conformation when solubilized in detergents. The goals of reconstitution can be summarized as follows. First, the protein must be inserted into a bilayer of desired lipid composition. This insertion is commonly achieved by solubilizing the lipid in detergent, mixing the solubilized lipid and protein together, and then removing the detergent by dialysis. This method produces LUVs containing various amounts of protein. Second, the reconstituted systems must have constant lipid to protein ratios between vesicles. Most reconstitution procedures give rise to heterogeneous systems, where vesicles contain various amounts of protein. Column chromatography techniques can be employed to obtain systems exhibiting uniform lipid to protein ratios (Madden, Hope, and Cullis 1984). Finally, the systems should have asymmetric protein orientation. In contrast with the intact biological membrane, the protein in reconstituted systems is not necessarily inserted with a well-defined asymmetric orientation. In the case of reconstituted cytochrome oxidase systems, for example, oxidase-containing vesicles can exhibit protein orientations in which the cytochrome *c* binding sites are on the outside or the inside. Asymmetric protein orientation can be achieved by reconstitution at low protein to lipid ratios such that most vesicles contain one or zero protein molecules. Populations containing only one oxidase molecule per vesicle with well-defined transmembrane orientations of the oxidase can subsequently be achieved by ion-exchange or affinity column chromatography, as illustrated in Figure 2.6.

In some cases asymmetric incorporation of other proteins can be achieved by different procedures. Erythrocyte glycoporphin, for example, has a large carbohydrate-containing region which is normally localized on the exterior of the red cell. Reconstituted systems can be obtained by hydrating a dried film of lipid and glycoporphin (McDonald and McDonald 1975), resulting in asymmetric vesicles in which more than 80% of the carbohydrate groups are on the vesicle exterior. This is presumably due to the small size of the reconstituted vesicle, which limits the fraction of the bulky carbohydrate-containing groups that can pack into the interior volume.

Alternative reconstitution techniques involving protein insertion into preformed vesicles have achieved some success in obtaining

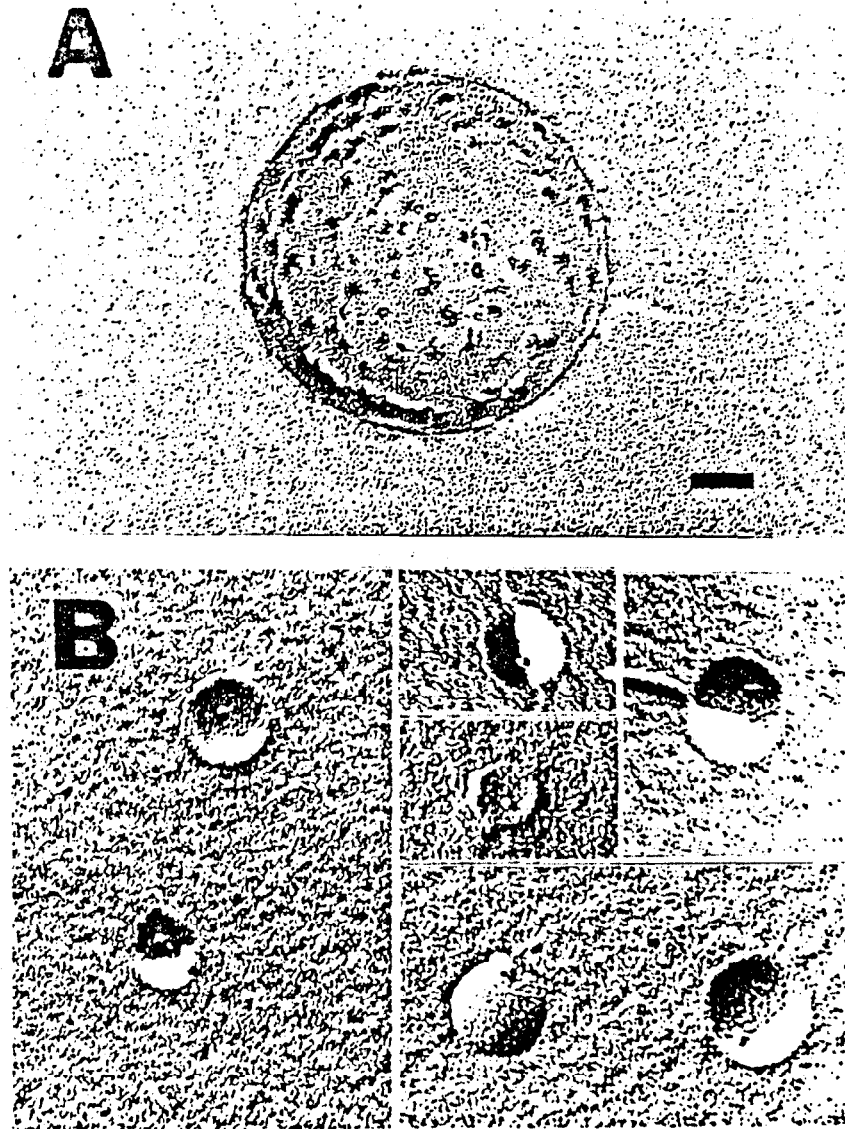


Figure 2.6. (A) Rotary-shadowed freeze-fracture micrograph of beef heart cytochrome c oxidase reconstituted into a vesicle of dioleoylphosphatidylcholine by the cholate dialysis procedure, at a protein to lipid ratio of 1:15 (w/w).

(B) Unidirectionally shadowed freeze-fracture micrographs of cytochrome c oxidase reconstituted at protein to lipid ratios of <math><1:5000</math> (w/w). Each particle has been shown to represent one dimer of cytochrome c oxidase and is approximately 10 nm in diameter. The bar represents 100 nm (see Madden, Hope, and Cullis [1984] for details of the reconstitution procedure).

asymmetric incorporation. One of these asymmetric insertion techniques utilizes the detergent octylglucoside. It is possible to form vesicles in the presence of relatively high detergent concentrations (approximately 20mM) which are sufficient to solubilize the spike protein of Semliki Forest virus (Helenius, Sarvas, and Simons 1981). The spike protein consists of a hydrophilic spike and a smaller hydrophobic anchor portion of the molecule. The anchor portion is solubilized by a coat of detergent, and this half of the molecule can insert into the preformed bilayer on dialysis.

In summary, a large variety of sophisticated and well-defined model membrane systems are becoming available. The simplest model systems consist of aqueous dispersions of lipid which can be converted to LUV forms by a variety of techniques. The subsequent incorporation of protein, with well-defined lipid to protein ratios and asymmetric transmembrane protein orientations, is becoming more feasible. Problems remain, however, both in removing the last traces of detergent in reconstituted systems and in generating the lipid asymmetry observed in biological membrane systems.

PHYSICAL
PROPERTIES OF
LIPIDS
Membrane Fluidity

It is common practice to characterize membranes in terms of an ill-defined parameter known as *fluidity*. Unfortunately, the concept of membrane fluidity can be most misleading. For example, it is commonly assumed that a more saturated lipid composition, or the presence of cholesterol, makes membranes less fluid. This is not necessarily the case. Strictly speaking, membrane fluidity is the reciprocal of membrane viscosity, which in turn is inversely proportional to rotational and lateral diffusion rates of membrane components. Thus, a linear relation between membrane fluidity and rotational and lateral diffusion rates would be expected with increasing cholesterol content, for example. However, incorporation of cholesterol into liquid-crystalline phosphatidylcholine model membranes has little or no influence on the phospholipid lateral diffusion rates (Lindblom, Johansson, and Arvidson 1981), and can actually increase rotational diffusion rates. The major influence of cholesterol or decreased unsaturation is to increase the order in the hydrocarbon matrix. It is this increase or decrease in order, which is a measurable quantity expressed by NMR or ESR order parameters (Davis 1983), for example, that should be correlated with such changes as increased or decreased membrane permeabilities.

Gel-Liquid-Crystalline
Phase Behavior

As indicated earlier, membrane lipids can exist in a frozen gel state or fluid liquid-crystalline state, depending on the temperature (Silvius 1982), as illustrated in Figure 2.7. Transitions between the gel and

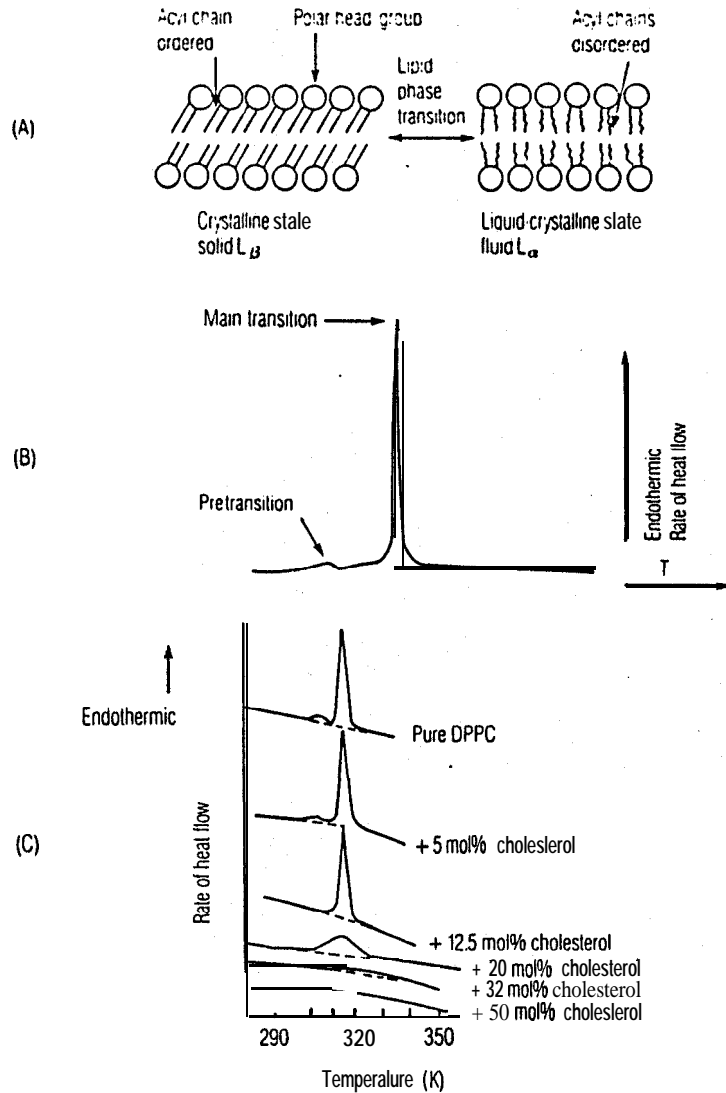


Figure 2.7. The phospholipid gel-liquid-crystalline phase transition and the effect of cholesterol. **(A)** Phospholipids, when fully hydrated, can exist in the gel or crystalline form (L_{β}) or in the fluid or liquid-crystalline state (L_{α}). In bilayers of gel-state phosphatidylcholine, the molecules can be packed such that the acyl chains are tilted with respect to the bilayer normal (L_{β}) state. Raising the temperature converts the crystalline state into the liquid-crystalline state. **(B)** The exothermic gel-liquid-crystalline phase transition as detected by DSC. T represents temperature. For dipalmitoylphosphatidylcholine (DPPC) the onset of the main transition occurs at approximately 41°C . The pretransition represents a small endothermic reorganization in the packing of the gel-state lipid molecules prior to melting. **(C)** Influence of cholesterol. The enthalpy of the phase transition (represented by the area under the endotherm) is dramatically reduced. At greater than 30 mol % cholesterol, the lipid phase transition is effectively eliminated. Adapted from Houslay and Stanley (1982).

liquid-crystalline phases can be monitored by a variety of techniques, including nuclear magnetic resonance (NMR), electron spin resonance (ESR), and fluorescence, among others. Perhaps the most direct technique is *differential scanning calorimetry (DSC)*, which measures the heat absorbed (or released) by a sample as it undergoes an endothermic (or exothermic) phase transition. A representative DSC scan of dipalmitoylphosphatidylcholine, which exhibits a gel to liquid-crystalline transition temperature (T_c) of 41°C , is illustrated in Figure 2.7. Three parameters of interest in such traces are the area under the transition peak, which is proportional to the enthalpy of the transition; the width of the transition, which gives a measure of the “cooperativity” of the transition; as well as the transition temperature T_c itself. The enthalpy of the transition reflects the energy required to melt the acyl chains, whereas cooperativity reflects the number of molecules that undergo a transition simultaneously.

Before describing the calorimetric behavior of various phospholipid systems, we emphasize two general points. First, gel-state lipids always assume an overall bilayer organization, presumably because the interactions between the crystalline acyl chains are then maximized. Thus, the nonbilayer hexagonal (H_{II}) or other phases discussed in the following section are not available to gel-state systems. Second, species of naturally occurring lipids exhibit broad noncooperative transitions due to the heterogeneity in the acyl chain composition. Thus, sharp gel-liquid-crystal transitions, indicating highly cooperative behavior, are observed only for aqueous dispersions of molecularly well-defined species of lipid. These can presently be obtained only by synthetic routes.

The calorimetric behavior of a variety of synthetic phospholipids is given in Table 2.5. There are three points of interest. First, for the representative phospholipid species, phosphatidylcholine, there is an increase in T_c by approximately 20°C as each two-carbon unit is added and a corresponding increase in enthalpy (2-3 kcal/mol). Second, inclusion of a cis double bond at C-9 results in a remarkable decrease in T_c , which is further lowered as the degree of unsaturation is increased. It is interesting to note that inclusion of only one cis-unsaturated fatty acid at the C-1 or C-2 position of the glycerol backbone is sufficient to lower T_c from 41°C for dipalmitoyl phosphatidylcholine to -5°C for the palmitoyl-oleoyl species, a major molecular subspecies of phosphatidylcholine in biological membranes. A final point is that the T_c and enthalpy are also sensitive to the head-group constituent. For example, molecular species of phosphatidylethanolamine commonly exhibit T_c values 20°C higher than corresponding species of phosphatidylcholine. The data of Table 2.5 have some predictive value in that approximate values of T_c can be estimated for other molecular species of lipids.

Table 2.5. Temperature (T_c) and Enthalpy (ΔH) of the Gel-Liquid-Crystalline Phase Transition of Phospholipids (in Excess Water)

Lipid species*	$T_c \pm 2^\circ\text{C}$	$\Delta H \pm 1 \text{ kcal/mol}$
12:0/12:0 PC	-1	3
14:0/14:0 PC	23	6
16:0/16:0 PC	41	8
16:0/18:1c Δ^9 PC		
16:1c Δ^9 /16:1c Δ^9 PC	-36	9
18:0/18:0 PC	54	10
18:1c Δ^9 /18:1c Δ^9 PC	-20	9
16:0/16:0 PE	63	9
16:0/16:0 PS	55	9
16:0/16:0 PC	41	9
16:0/16:0 PA	67	5

* The code denotes the number of carbons per acyl chain and the number of double bonds. Δ gives the position of the double bond. The abbreviations are PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; c indicates cis.

The calorimetric behavior of individual lipid species cannot be directly related to the behavior of the complex lipid mixtures found in biological systems; therefore, considerable attention has been devoted to the properties of mixtures of pure lipid species. Two general features have emerged. First, when all component lipids are liquid crystalline (that is, $T > T_c$), the lipid systems exhibit characteristics consistent with complete mixing of the various lipids. Second, at temperatures below the T_c of one of the constituents, separation of the component with the highest melting temperature into crystalline domains (*lateral phase separation*) can occur under certain conditions. For example, equimolar mixtures of two saturated phosphatidylcholines differing by four carbon units or more ($\Delta T_c > 20^\circ\text{C}$) can exhibit lateral phase separation (indicated by calorimetric and freeze-fracture studies). On the other hand, mixtures of two phosphatidylcholines differing in their length by only two carbon units are miscible and co-crystallize at a temperature intermediate between the component T_c values. Similar behavior is observed for binary mixtures of synthetic lipids with different head group compositions-if the T_c values of the components differ by more than 20°C , lateral phase separation phenomena can occur. It should be **emphasized that** lateral segregation of a particular lipid species in the plane of the membrane has been observed only for model systems in which a sizable proportion of the lipids exhibit a T_c which is not only well above the temperature at which the experiment is performed but is also significantly greater than the T_c of other component lipids.

Further studies of the calorimetric behavior of lipid systems have emphasized the remarkable physical properties of cholesterol (Demel and de Kruijff 1976), which are detailed in part in the previous chapter. This lipid has the ability to inhibit the crystallization of lipids to form gel-state systems, as illustrated for dipalmitoylphosphatidylcholine in Figure 2.7. The enthalpy of the transition is progressively reduced as the cholesterol content is increased. For phosphatidylcholine to cholesterol molar ratios of 2:1, no transition is observable. This lipid mixture exhibits basically liquid-crystalline characteristics, since, as indicated previously, equimolar cholesterol levels do not reduce the lateral diffusion rates of (liquid-crystalline) phosphatidylcholine significantly (the lateral diffusion rates of gel-state lipids are at least two orders of magnitude slower). Furthermore, at temperatures below the T_c of the phospholipid, NMR characteristics indicative of rapid axial rotation of the phospholipid molecule are observed in the presence of cholesterol, behavior similar to that observed for liquid-crystalline phospholipids.

The relation between the gel-liquid-crystalline properties of lipids and the roles of lipids in biological membranes remains obscure. The observation that individual lipid components can adopt gel or liquid-crystalline arrangements has led to the suggestion that segregation of particular lipids into a local gel-state environment may occur within a biological membrane. This segregation could affect protein function by restricting protein mobility within the bilayer matrix or could provide packing defects resulting in permeability changes. There are two major difficulties encountered with these concepts, however. First, while certain procaryotic systems can exhibit characteristics consistent with the presence of gel-state lipids at temperatures which allow growth, such observations are by no means universal. In eucaryotic membranes, for example, there is no evidence for the presence of gel-state lipid components at physiological temperatures. The second difficulty concerns the way in which lateral segregation of lipid into crystalline domains might be regulated. Clearly, an organism cannot regulate fluidity by regulating temperature; thus, physiological factors are required which can induce isothermal modulation of the local lipid composition. The presence of factors capable of segregating lipids into local crystalline domains in a biological membrane has not been unambiguously demonstrated.

The theme that membranes do not require the presence of gel-state lipids is easily developed for eucaryotic membrane systems, such as the well-characterized erythrocyte membrane. Of the erythrocyte membrane lipids, only sphingomyelin exhibits a T_c close to physiological temperatures, with the attendant possibility of forming local crystalline domains. However, this possibility is seriously compromised by the presence in the membrane of equimolar levels of

cholesterol, which would be expected to inhibit such formation, in agreement with the observation that no reversible phase transition is observable in the intact erythrocyte (ghost) membrane by calorimetric or other techniques. In other membranes which contain little or no cholesterol, such as the membranes of various subcellular organelles, the absence of gel-state domains is indicated **by** the absence of relatively saturated lipid species, such as **sphingomyelin**, as well as by the increased unsaturation of other lipids **present**. Table 2.3 gives the unsaturation index (number of unsaturated bonds per **phospholipid**) for the lipid component of a variety of membranes. In general, more metabolically active membranes, such as the inner **mitochondrial** membrane, contain a higher fraction of more unsaturated lipid species (whose T_c values may be 60°C or more below physiological temperatures).

In summary, available evidence indicates that membranes require a fluid **bilayer** matrix for function and that modulation of local fluidity and function by formation of **crystalline** domains is unlikely to be a general phenomenon.

Lipid Polymorphism In addition to an ability to adopt a gel or liquid-crystalline **bilayer** organization, lipids can also adopt entirely different liquid-crystalline structures on hydration (Cullis et al. 1983). The major structures assumed are illustrated in Figure 2.8. These structures have three general features. First, the predominant structures assumed by isolated species of membrane lipids on hydration in excess aqueous buffer are the familiar bilayer organization and the **hexagonal H_{II}** structure. Lipids which form **micellar** structures, such as **lysophosphatidylcholine**, are minority components of membranes. Second, the **H_{II}** phase, which consists of a hydrocarbon matrix penetrated by hexagonally packed aqueous cylinders with diameters of about 20 Å, is not compatible with maintenance of a permeability barrier between external and internal compartments. This immediately raises questions concerning the functional role of lipids in membranes which preferentially adopt this structure in isolation. Finally, in contrast with the situation for gel-state (crystalline) lipids, it now appears that **all** biological membranes contain an appreciable fraction (up to 40 **mol %**) of **lipid** species which prefer the **H_{II}** arrangement, as **well** as **lipids** which prefer bilayer structure.

The ability of lipids to adopt different structures on hydration is commonly referred to as **lipid** polymorphism. Three techniques which have been extensively employed to monitor lipid polymorphism are X-ray diffraction, **^{31}P** - and **^2H** -NMR, and freeze-fracture procedures. X-ray diffraction is the classical technique, allowing the detailed nature of the phase structure to be elucidated. The use of **^{31}P** -NMR for

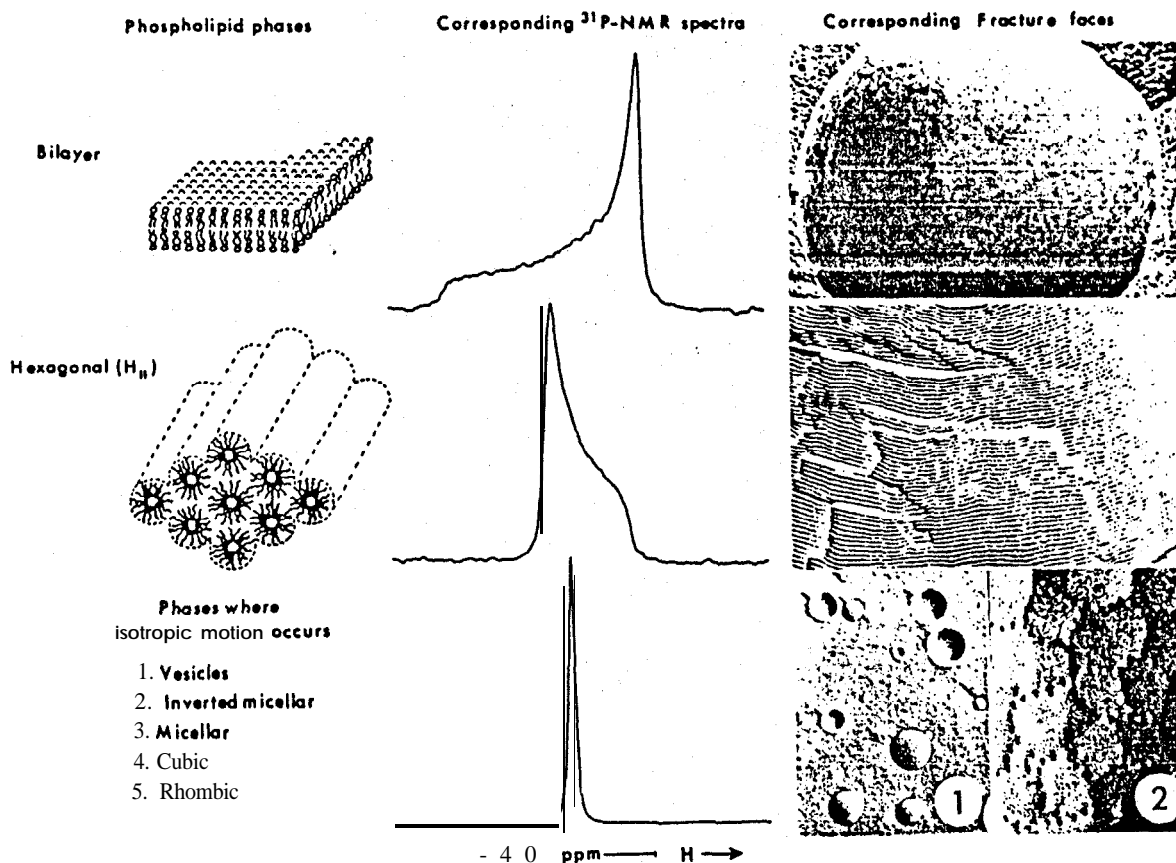


Figure 2.8. ^{31}P -NMR and freeze-fracture characteristics of phospholipids in various phases. The bilayer spectrum was obtained from aqueous dispersions of egg yolk phosphatidylcholine, and the hexagonal (H_{II}) phase spectrum from phosphatidylethanolamine (prepared from soybean phosphatidylcholine). The ^{31}P -NMR spectrum representing isotropic motion was obtained from a mixture of 70 mol % soy phosphatidylethanolamine and 30 mol % egg yolk phosphatidylcholine. All preparations were hydrated in 10mM Tris-acetic acid (pH 7.0) containing 100mM NaCl, and the spectra were recorded at 30°C in the presence of proton decoupling. The freeze-fracture micrographs represent typical fracture faces obtained. The bilayer configuration (total erythrocyte lipids) gives rise to a smooth fracture face, whereas the hexagonal (H_{II}) configuration is characterized by ridges displaying a periodicity of 6-15 nm. Two common conformations that give rise to isotropic motion are represented in the bottom micrograph: (1) bilayer vesicles (less than 200 nm diameter) of egg phosphatidylcholine prepared by extrusion techniques and (2) large lipid structures containing lipidic particles (egg phosphatidylethanolamine containing 20 mol % egg phosphatidylserine at pH 4.0).

identification of polymorphic phase characteristics of phospholipids relies on the different motional averaging mechanisms available to phospholipids in different structures and provides a convenient and

reliable diagnostic technique. Finally, freeze-fracture electron microscopy allows visualization of local structure which need not be arranged in a regular lattice, yielding information not available from X-ray or NMR techniques.

The ^{31}P -NMR and freeze-fracture characteristics of bilayer and H_{II} phase phospholipid systems are illustrated in Figure 2.8. Bilayer systems exhibit broad, asymmetric ^{31}P -NMR spectra with a low-field shoulder and high-field peak separated by about 40 ppm, whereas H_{II} phase systems exhibit spectra with reversed asymmetry which are narrower by a factor of two. The difference between bilayer and H_{II} phase ^{31}P -NMR spectra arises from the ability of H_{II} phase phospholipids to diffuse laterally around the aqueous channels (Cullis et al. 1983). Freeze-fracture techniques show flat, featureless fracture planes for bilayer systems, whereas H_{II} phase structures give rise to a regular corrugated pattern as the fracture plane cleaves between the hexagonally packed cylinders.

The polymorphic phase preferences of a large variety of synthetic and naturally occurring phospholipids have been investigated, and the results obtained for eucaryotic lipid species are summarized in Table 2.6. It is immediately apparent that a significant proportion of membrane lipids adopt or promote H_{II} phase structure under appropriate conditions. Phosphatidylethanolamine, which commonly comprises up to 30% of membrane phospholipids, is perhaps

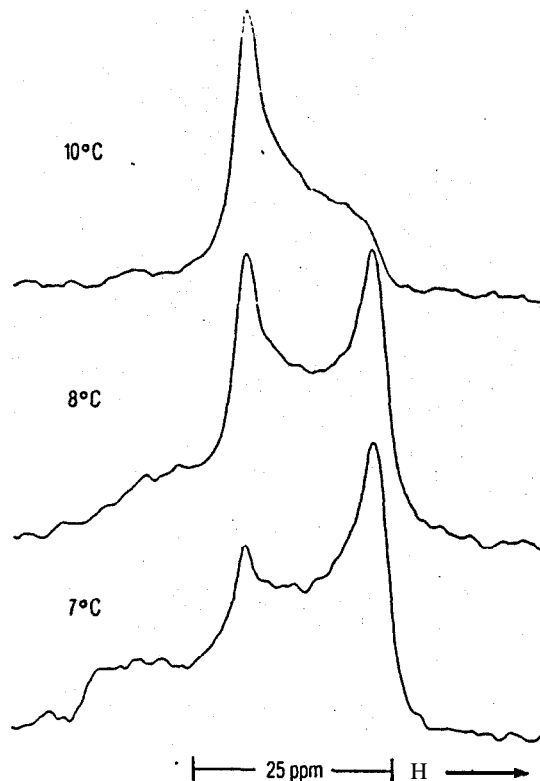
Table 2.6. Phase Preferences of Membrane Lipids from Eucaryotes

<i>Bilayer</i>	<i>Hexagonal H_{II}</i>
PC	
SPM	
	PE
PS	PS (pH < 3)
PG	
PI	
PA	PA (+Ca ²⁺)
	PA (pH < 3)
CL	CL (+Ca ²⁺)
	Cholesterol*
	Fatty acids

Note: The abbreviations are PC, phosphatidylcholine; SPM, sphingomyelin; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine.

* Cholesterol and long-chain unsaturated fatty acids can induce the hexagonal (H_{II}) phase in some lipid mixtures.

Figure 2.9. 36.4-MHz ^{31}P -NMR spectra of an aqueous dispersion of human erythrocyte phosphatidylethanolamine dispersed in 25mM Tris-acetic acid (pH 7.0) and 2mM EDTA. These spectra were obtained employing broad-band proton decoupling.



the most striking example, and particular effort has been devoted to understanding the factors which result in a **predilection** for the H_{II} arrangement. As illustrated in Figure 2.9, **phosphatidylethanolamine** isolated from erythrocytes can adopt both the **bilayer** and H_{II} arrangements, depending on the temperature. The H_{II} structure is **formed** above a characteristic bilayer to hexagonal (H_{II}) **transition** temperature (T_{BH}) of about 10°C . Similar or lower values of T_{BH} have been observed for phosphatidylethanolamine isolated **from endoplasmic reticulum** and the inner **mitochondrial** membrane. **Lower T_{BH} values** are observed for more unsaturated species. This dependence of T_{BH} on acyl chain unsaturation has been character&d **more definitively**, employing synthetic species of **phosphatidylethanolamine**, as summarized in Table 2.7. This table illustrates **that a minimal degree** of unsaturation of the acyl chains is required for H_{II} **structure** to be adopted and that increased unsaturation **progressively favors the H_{II} arrangement**.

Biological membranes contain **mixtures** of lipids **which individually** prefer bilayer or H_{II} structures; therefore, the **properties of** mixed systems are of considerable interest. Studies on **model systems**

Table 2.7. The Temperature (T_{BH}) of the Bilayer-Hexagonal H_{II} Phase Transition for Some Phosphatidylethanolamines

Physical Properties of Lipids

Phosphatidylethanolamine	T_{BH} (°C)
18:0/18:0	>105
18:1 Δ^9 /18:1 Δ^9	60 to 63
18:1 $c\Delta^9$ /18:1 $c\Delta^9$	10
18:2 $c\Delta^{9,12}$ /18:2 $c\Delta^{9,12}$	-15 to -25
18:3 $c\Delta^{9,12,15}$ /18:3 $c\Delta^{9,12,15}$	-15 to -30

show that mixtures of an H_{II} phase lipid (for example, phosphatidylethanolamine) with a bilayer phospholipid (such as phosphatidylcholine) result in a progressive stabilization of net bilayer structure for the whole mixture as the percentage of bilayer lipid increases, as illustrated in Figure 2.10. This is a general feature of mixtures of

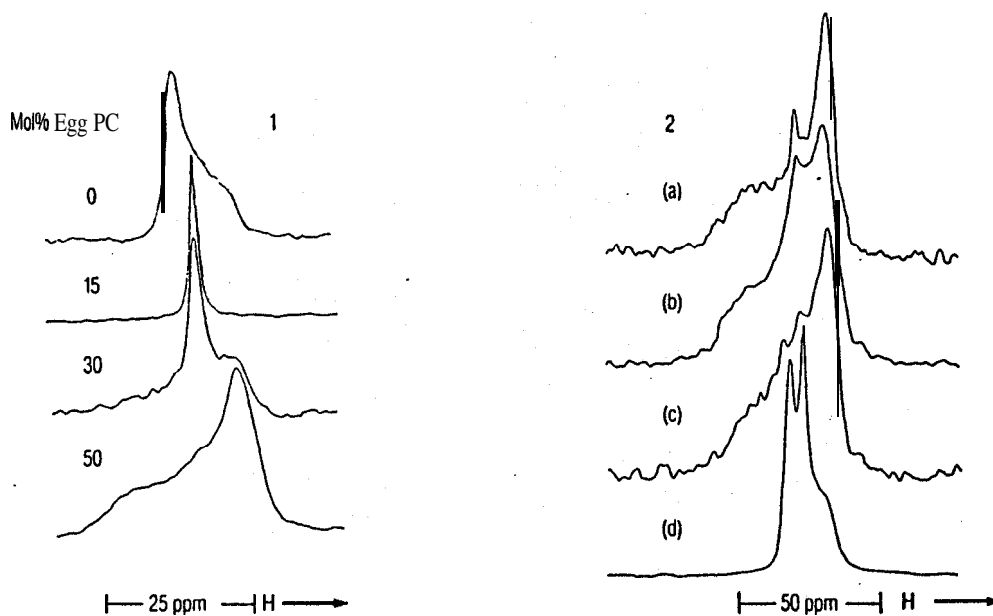


Figure 2.10. Phase behavior of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) mixtures and the effects of cholesterol. (1) 36.4-MHz ^{31}P -NMR spectra of aqueous dispersions of mixtures of soy phosphatidylethanolamine and egg phosphatidylcholine. The amount present is expressed as a percentage of the total phospholipid. (2) 36.4-MHz ^{31}P -NMR spectra obtained at 30°C from equimolar mixtures of dioleoyl-PE with dioleoyl-PC in the presence of (a) 0 mol %; (b) 15 mol %; (c) 30 mol %, and (d) 50 mol % cholesterol.

bilayer and H_{II} lipids. Depending on the acyl chain composition, temperature, and head group size and charge, complete bilayer stabilization can be achieved by the addition of 10 to 50 mol % of the bilayer species. These systems appear to retain the ideal mixing behavior characteristic of liquid-crystalline systems. For example, in phosphatidylethanolamine-phosphatidylcholine mixtures containing intermediate amounts of the bilayer-stabilizing species, situations can arise where H_{II} phase and bilayer phase components coexist in the same sample. 1H -NMR studies of 1H -labeled varieties of these lipids indicate a homogeneous lipid composition, with no preference of the H_{II} -preferring phosphatidylethanolamine species for the H_{II} component or of phosphatidylcholine for the bilayer component (Tilcock et al. 1982).

There are two other features of these mixed systems which are of particular interest. The first concerns cholesterol, which has the remarkable ability to induce H_{II} phase structure for phosphatidylethanolamine-containing systems where bilayer structure has been stabilized by phosphatidylcholine (Figure 2.10). This effect of cholesterol is also observed in other mixed-lipid systems (Tilcock et al. 1982). The second point concerns the narrow ^{31}P -NMR peak occasionally observed in the mixed-lipid systems of Figure 2.10. Such a spectral feature arises from phospholipids, which experience *isotropic motional averaging* over all possible orientations. Such a resonance cannot arise from phospholipids in H_{II} or large (diameter ≥ 200 nm) bilayer structures, where the motion is restricted. **Freeze-fracture** studies suggest that this isotropic peak corresponds to a novel particulate feature observed on the fracture face of these systems, as illustrated in Figure 2.11. These "lipidic particles" are a general feature of mixtures of bilayer- and H_{II} -preferring lipids (Verkleij 1984), and, there is a growing consensus that they correspond to inverted micellar **interbilayer** structures, as indicated in Figure 2.11. These structures appear to represent intermediaries between the bilayer and H_{II} phases and may be of particular importance, as such nonbilayer structures can be localized to a particular region of the membrane. Such structures may have functional utility, since their formation would not result in a large-scale disruption of the bilayer permeability barrier that necessarily accompanies generation of macroscopic H_{II} phase lipid structure.

The functional roles of nonbilayer lipid structures in membranes have been investigated by characterizing the influence of **divalent** cations, ionic strength, **pH**, and membrane protein on lipid polymorphism (Cullis et al. 1983). These factors can strongly influence the structural preferences of appropriate lipid systems. In the case of pure lipid systems, for example, reduction of the **pH** results in H_{II} phase structure for (unsaturated) phosphatidylserine and

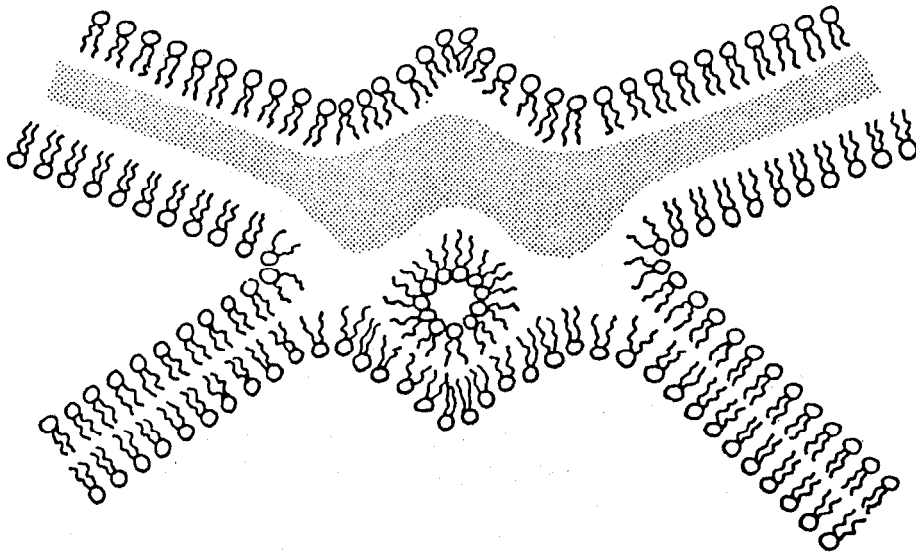
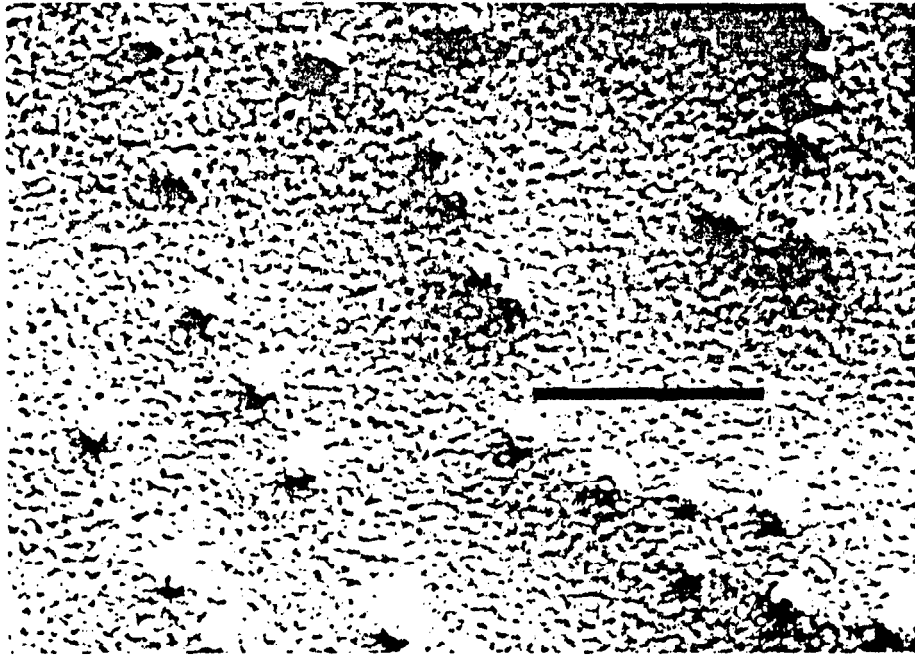


Figure 2.11. A schematic representation of the proposed fracture plane around an inverted micelle formed at the contact point between **two** bilayers. The corresponding lipidic particles observed by freeze-fracture electron microscopy are shown in the micrograph of a mixture of soy phosphatidylinositol(7.5 mol %) with soy phosphatidylethanolamine. Each particle is approximately 10 nm in **diameter**. The scale represents 100 nm.

phosphatidic acid systems, and the addition of Ca^{2+} to cardiolipin **triggers bilayer- H_{II} transitions** (Table 2.6). Similar observations extend to mixed-lipid systems, where the addition of Ca^{2+} to bilayer systems containing phosphatidylethanolamine and various acidic phospholipids can also trigger H_{II} phase formation.

Phosphatidylserine-phosphatidylethanolamine systems are perhaps the best characterized in this regard (Tilcock et al. 1984), and certain features of the mechanisms involved deserve emphasis. First, in some binary phospholipid mixtures containing **phosphatidylserine**, Ca^{2+} can segregate the phosphatidylserine component into a crystalline (gel-phase) structure with a characteristic morphology described as *cochleate* (as observed by freeze-fracture). In the case of phosphatidylserine-phosphatidylethanolamine systems, the bilayer-stabilizing influence of phosphatidylserine is thus removed, allowing the phosphatidylethanolamine to adopt the H_{II} organization it favors in isolation. When cholesterol is present, however, Ca^{2+} -dependent generation of H_{II} structure proceeds by a different mechanism which does not involve lateral segregation **phenomena**—rather, all lipid components, including phosphatidylserine, adopt the H_{II} organization. The potential relevance of these observations is illustrated by Figure 2.12, where it is shown that Ca^{2+} can trigger H_{II} formation in a mixture of lipids isolated from human erythrocytes, with a composition corresponding to that of the erythrocyte inner monolayer (which contains predominantly **phosphatidylethanolamine** and phosphatidylserine).

The ability of lipids to adopt different macroscopic structures on hydration has stimulated studies aimed at understanding the physical properties of lipids which dictate these preferences. These studies have given substantial support to a simplistic hypothesis that a generalized *shape property* of lipids determines the phase structure adopted (Cullis et al. 1983). This concept is illustrated in Figure 2.13, where bilayer phase lipids are proposed to exhibit cylindrical geometry compatible with that organization, while H_{II} phase lipids have a cone shape where the acyl chains subtend a larger cross-sectional area than the polar head group region. Detergent-type lipids which form micellar structures are suggested to have reversed geometry corresponding to an inverted cone shape. It should be noted that “shape” is an inclusive term reflecting the effects of the size of polar and **apolar** regions, head group hydration and charge, hydrogen-bonding processes, and effects of counterions, among other possibilities. The cone shape of unsaturated phosphatidylethanolamines, for example, can be ascribed to a smaller, less-hydrated head group (in comparison with phosphatidylcholine). There may also be intermolecular hydrogen bonding between phosphatidylethanolamines, which would further reduce the area per molecule in the head **group** region.

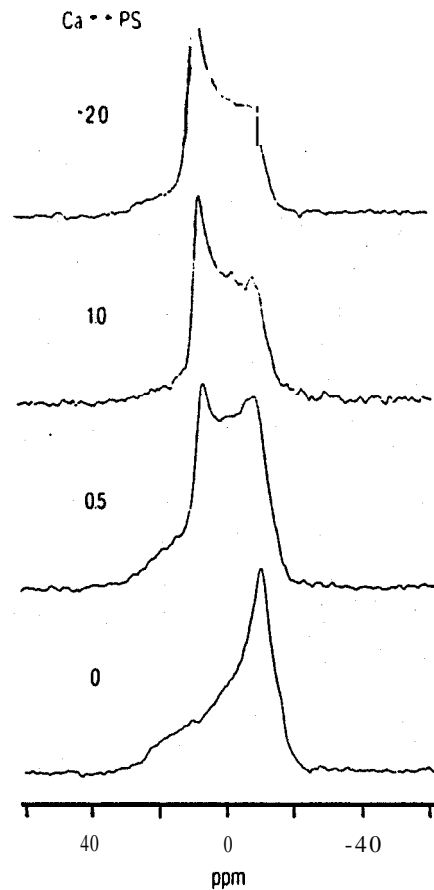


Figure 2.12. Effect of calcium on "inner monolayer" phospholipids: **81.0-MHz ^{31}P -NMR** spectra at 37°C arising from an aqueous dispersion of reconstituted inner monolayer lipid isolated from human **erythrocyte** membrane. The lipid composition is **PE:PS:PC:SPM** (in the ratios **0.5:0.25:0.13:0.12**) and contains equimolar cholesterol with respect to total phospholipid. The ratio **Ca^{2+}/PS** refers to the molar ratio of **Ca^{2+}** to PS.

Alternatively, the increased predilection of more unsaturated species of phosphatidylethanolamine for the **H_{II}** arrangement (Table 2.7) may be attributed to the increased cross-sectional area of the unsaturated (compared with saturated) acyl chains. A striking observation supporting the shape concept is that lipid mixtures containing detergents (inverted cone shape) and unsaturated **phosphatidylethanolamines** (cone shape) can adopt bilayer structure, which may be attributed to shape **complementarity** (Cullis et al. 1983).

In summary, studies on model systems show that **lipids** found in biological membranes can exist in a variety of structures in addition to

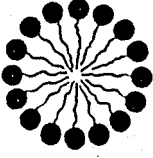

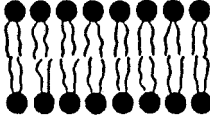

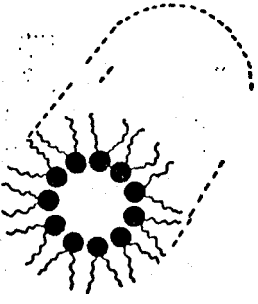
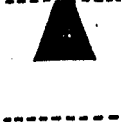
LIPID	PHASE	MOLECULAR, SHAPE
LYSOPHOSPHOLIPIDS DETERGENTS	 MICELLAR	 INVERTED CONE
PHOSPHATIDYLCHOLINE SPHINGOMYELIN PHOSPHATIDYLSERINE PHOSPHATIDYLINOSITOL PHOSPHATIDYLGlycerol PHOSPHATIDIC ACID CARDIOLIPIN DIGALACTOSYLDIGLYCERIDE	 BILAYER	 CYLINDRICAL
PHOSPHATIDYLETHANOLAMINE (UNSATURATED) CARDIOLIPIN - Ca^{2+} PHOSPHATIDIC ACID - Ca^{2+} (pH < 6.0) PHOSPHATIDIC ACID (pH < 3.0) PHOSPHATIDYLSERINE (pH < 4.0) MONOGALACTOSYLDIGLYCERIDE	 HEXAGONAL (H_{II})	 CONE

Figure 2.13. Polymorphic phases and corresponding dynamic molecular shapes of lipids.

the bilayer phase. These structural preferences can be modulated by many biologically relevant variables, supporting the possibility that nonbilayer lipid structures play roles in membrane-mediated phenomena requiring local departures from bilayer organization. As indicated later in this chapter, membrane fusion may be a most important example.

LIPIDS AND THE PERMEABILITY PROPERTIES OF MEMBRANES

The ability of lipids to provide a bilayer permeability barrier between external and internal environments constitutes one of their most important functions in a biological membrane. The nature and selectivity of this barrier to various molecules and ions of biological interest (water, uncharged water-soluble nonelectrolytes, and ionized solutes of varying hydrophobicity) have been extensively investigated. A succinct review of this data is difficult to achieve, due in part to the different model systems employed and the discrepancies among experiments. One major problem concerns the use of the black lipid membrane or LUV systems where residual solvents such as decane, detergent, ether, or ethanol may influence the permeability properties of the ion of interest. Here we present a summary of salient general principles of membrane permeability in relation to properties of component lipids such as fluidity, polar head group charge, and phase structure. A synopsis of the permeability coefficients observed for different solutes for a variety of membranes is presented in Table 2.8.

Table 2.8. Permeability Coefficients (cm/s) for Some Common Polar Solutes Across Model and Biological Membranes (at 20°C unless otherwise indicated)

Membrane	Na ⁺	K ⁺	Cl ⁻
Egg phosphatidylcholine (4°C)	<1.2 × 10 ⁻¹⁴	—	5.5 × 10 ⁻¹¹
Soy phosphatidylcholine [†]	4.0 × 10 ⁻¹³	—	7.0 × 10 ⁻¹¹
Equimolar soy phosphatidyl- [*] choline and cholesterol (40°C)	<2.0 × 10 ⁻¹³	—	—
Dilinoleoylphosphatidylserine [*]	<7.0 × 10 ⁻¹³	—	—
Human red blood cell [*] phosphatidylserine	<7.0 × 10 ⁻¹³	—	—
Human red blood cell [†]	1.0 × 10 ⁻¹⁰	2.4 × 10 ⁻¹⁰	1.4 × 10 ⁻⁴
Squid axon (resting) [†]	1.5 × 10 ⁻⁸	5.6 × 10 ⁻⁸	1.0 × 10 ⁻⁸
Squid axon (excited) [†]	5.0 × 10 ⁻⁶	1.7 × 10 ⁻⁴	1.0 × 10 ⁻⁸

^{*} The data were obtained from large unilamellar vesicles prepared by extrusion through filters according to Hope et al. (1985).

[†] The data were adapted from Jain and Wagner (1980).

Theoretical Considerations In order to appreciate the meaning of the permeability coefficient parameter for a given lipid system, some understanding of the underlying theory is required. A basic phenomenological treatment of diffusion begins with *Fick's law*, which states that the diffusion rate of a given substance (number of molecules per unit time, dn/dt) through a membrane is directly proportional to the area A of the membrane and the difference in the concentration $\Delta C(t)$ of the material across the membrane. Thus, $dn/dt \propto A\Delta C(t)$, which may be rewritten as $dn/dt = -PA\Delta C(t)$, where P , which has the units of length over time (for example, cm/s), is the permeability coefficient and t is time. If we consider the special case of a LUV of radius R containing an initial concentration of solute $C_i(0)$, where the initial external concentration of this solute is zero, it is straightforward to show that $\Delta C(t) = C_i(0)\exp(-3Pt/R)$. Under conditions where the external volume is much greater than the internal trapped volume, $\Delta C(t) \cong C_i(t)$ (where $C_i(t)$ is the internal concentration at time t); thus, $C_i(t) = C_i(0)\exp(-3Pt/R)$. For a 100-nm diameter LUV it may therefore be calculated that the time required for release of one-half the entrapped material ($t_{1/2}$) is 0.1 s for $P = 10^{-5}$ cm/s, whereas for $P = 10^{-10}$ cm/s, $t_{1/2} = 3.2$ h.

It should be emphasized that the preceding example, while illustrative, neglects several important factors which can strongly influence the net flux of molecules through membranes. These include the effects associated with the aqueous layer (more than 20 nm thick) that extends from the lipid-water interface, in which solute molecules are not mixed to the same extent as in the bulk solution (Fettiplace and Haydon 1980). Such unstirred layers can effectively reduce the solute concentration difference ΔC across the membrane itself, giving rise to a smaller measured value of P . For charged molecules, the **efflux** can be strongly limited by generation of a membrane potential, as will be discussed later. Finally, the permeability of various solutes through membranes is strongly temperature dependent, with activation energies E_0 in the range of 8-20 kcal/mol. A measure of the influence of temperature is given by the observation that an activation energy of 12 kcal/mol will increase the permeability **coefficient** by a factor of two for every 10°C increase in temperature.

Permeability of Water and Nonelectrolytes

Liquid-crystalline lipid bilayers are remarkably permeable to water, which exhibits permeability coefficients in the range of 10^{-2} to 10^{-4} cm/s (Fettiplace and Haydon 1980). Membrane systems **enclosing** high concentrations of a relatively impermeable solute **will** swell **when** placed in an aqueous medium containing little or no solute, due to a net influx of water to achieve osmotic balance. Conversely, the reverse conditions will lead to shrinkage. As a result, the **relative**

permeability of different membrane systems to water can be monitored by measuring swelling rates (employing light scattering techniques, for example) when osmotic gradients are applied (Blok, van Deenen, and de Cier 1976). Results obtained from such studies indicate that increased unsaturation of the fatty acids of the membrane causes increases in water permeability. Similarly, the inclusion of cholesterol reduces water permeability, leading to the general conclusion that factors contributing to increased order in the hydrocarbon region decrease water permeability. It is of interest to note that whereas **MLVs** and **LUVs** in the size range of 1 μm or larger demonstrate these osmotic properties, the smaller **SUV** systems do not.

The diffusion properties of nonelectrolytes (uncharged polar solutes) appear to depend on the properties of the lipid matrix in much the same manner as does the diffusion of water. In general, the permeability coefficients observed are at least two orders of magnitude smaller. For example, the permeability coefficient of urea across egg phosphatidylcholine bilayers is approximately 4×10^{-6} cm/s at 25°C (Poznansky et al. 1976). Furthermore, for a given homologous series of compounds, the permeability increases as the solubility in a hydrocarbon environment increases, indicating that the rate-limiting step in diffusion is the initial partitioning of the molecule into the lipid bilayer (Poznansky et al. 1976). With regard to the influence of lipid composition on the permeability of nonelectrolytes, the order in the acyl chain region has the same qualitative effects as in the case of water. Thus, decreased unsaturation of lipids or increased cholesterol content results in lower permeability coefficients (**Fettiplace and Haydon** 1980). Gel-phase systems are particularly impermeable. However, in systems exhibiting lateral phase separation of gel and liquid-crystalline domains, the permeability can be higher than for liquid-crystalline systems. This increased permeability can be attributed to packing defects at the crystalline-liquid-crystalline hydrophobic interface.

Permeability of Ions Lipid bilayers are remarkably impermeable to most small ions (Table 2.8). Permeability coefficients of less than 10^{-10} cm/s are commonly observed, and they can be as small as 10^{-14} cm/s for **Na⁺** and **K⁺**. For the example of a 100-nm diameter LW, this would correspond to a half-life for release of entrapped **Na⁺** of approximately 3.6 years. In contrast, lipid bilayers appear to be much more permeable to **H⁺** or **OH⁻** ions, which have been reported to have permeability coefficients in the range of 10^{-4} cm/s (Deamer 1982). The **Cl⁻** anion also exhibits anomalous permeability behavior, with permeability coefficients up to 300 times greater than those observed for **Na⁺** in similar systems.

Measures of the permeability of membranes to small ions are complicated, since for free permeation to proceed, a counterflow of other ions of equivalent charge is required; otherwise, a membrane potential is established which is equal and opposite to the chemical potential of the diffusing species. As an example, consider the 100-nm diameter LUV which has a well-buffered interior pH of 4.0 and an exterior pH of 7.0 in a Na⁺ buffer. The relatively permeable H⁺ ions can diffuse out, but Na⁺ ions cannot move in. Thus, a membrane potential ($\Delta\psi$) is established (interior negative), where

$$\Delta\psi = -59 \log \frac{[\text{H}^+]_i}{[\text{H}^+]_o} = -177 \text{ mV}$$

and the subsequent efflux of protons is coupled to the much slower influx of Na⁺ ions. Assuming a membrane thickness of 4 nm and interior dielectric constant of 2, the capacitance of the vesicle membrane can be calculated as $C = 0.5 \mu\text{F}/\text{cm}^2$; thus, the number of protons that diffuse out to set up $\Delta\psi$ can be calculated to be about 150. Subsequent H⁺ efflux will occur only as Na⁺ ions permeate in.

The relation between the physical properties of lipids and the permeability properties of membranes to small ions is not understood in detail. Difficulties in understanding this relationship arise from the different model systems employed, the various impurities present, and complexities due to ion counterflow and related membrane potential effects. Vesicles prepared by techniques involving detergents or organic solvents contain residual detergent or solvent which can strongly influence the permeabilities observed, and the presence of *n*-decane or other long chain alkanes in black lipid membrane systems may also influence permeability. In general, however, the permeability of a given ion appears to be related to the order in the hydrocarbon region, where increased order leads to a decrease in permeability.

The charge on the phospholipid polar head group can also strongly influence permeability by virtue of the resulting surface potential ϕ . For example, approximately 30% of the lipid of the inner monolayer of the erythrocyte membrane is the negatively charged lipid phosphatidylserine. If we assume an area per lipid molecule of 0.6 nm^2 , the resultant surface charge density σ is $8 \mu\text{C}/\text{cm}^2$ (where C is coulombs). The resulting surface potential ϕ can be calculated from Gouy-Chapman theory (McLaughlin 1977) for a 150mM monovalent salt buffer according to the relation $\phi = 0.052 \times \sinh^{-1}(\sigma/4.5)$. This gives a negative surface potential of $\phi = -69 \text{ mV}$. This potential will repel anions from and attract cations to the lipid-water interface. For example, the H⁺ concentration at the inner monolayer interface will be increased by the Boltzmann factor $\exp(e\phi/kT) = 14.5$ in compari-

son with the bulk solution, resulting in a significantly lower pH at the membrane interface and correspondingly higher H^+ efflux rates.

LIPID-PROTEIN INTERACTIONS Any complete understanding of biological membrane systems necessitates a detailed understanding of the nature and influence of lipid-protein interactions. Such interactions can be divided into two classes. The first concerns proteins with hydrophobic segments which penetrate into or through the lipid bilayer (*intrinsic*, or *integral*, *proteins*), whereas the second concerns water soluble proteins which interact electrostatically with negatively charged groups at the lipid-water interface (*extrinsic*, or *peripheral*, *proteins*). The effects of intrinsic and extrinsic proteins on membrane lipid fluidity (for example, the gel or liquid-crystalline nature of associated lipids) or lipid polymorphism will provide the primary focus of this section. However, it should be noted that studies of lipid-protein interactions (Jost and Griffith 1982) have generated a large and often confusing literature which has not yet led to a generally accepted understanding. We emphasize here only those points which we believe provide the most important insight.

Extrinsic Proteins The interaction of extrinsic proteins with lipids has been studied using a variety of proteins, including polylysine, cytochrome c, the A_1 basic protein from myelin, and spectrin from the red blood cell. In order for these basic (positively charged) molecules to interact extensively with lipid systems, the presence of acidic (negatively charged) lipids is required, consistent with an electrostatic protein-membrane association. Two general points can be made. First, while it is possible that such surface interactions may induce a time-averaged enrichment of the negatively charged lipid in the region of the protein, there is presently no unambiguous evidence to suggest that such clustering can induce a local fluidity decrease via formation of crystalline domains. Indeed, in model membrane systems containing acidic phospholipids, such extrinsic proteins as cytochrome c, the A_1 basic protein, and spectrin induce a decreased T_c and enthalpy of the lipid gel-liquid-crystalline transition, indicating an increased disorder in the acyl chain region. This effect has been related to an ability of such proteins to partially penetrate the hydrophobic region, as indicated by increases in permeability and monolayer surface pressure on binding. The second point is that there is evidence of competition between **divalent** cations and extrinsic proteins for binding to membranes. Thus, spectrin can shield the effects of Ca^{2+} on the gel-liquid phase transition properties of systems containing negatively charged lipids.

Studies on the influence of extrinsic protein on the polymorphic properties of lipids (de Kruijff et al. 1984) also yield results consistent with a competition between the protein and **divalent** cations. For example, polylysine, which is highly positively charged, can to some extent destabilize the bilayer structure of cardiolipin-phosphatidylethanolamine systems and strongly protects against the ability of Ca^{2+} to induce complete **H_{II}** organization in the pure lipid system. A particularly interesting observation is that cytochrome c can induce nonbilayer structures in cardiolipin-containing systems. This observation may be related to an apparent ability of **cytochrome c** to **translocate** rapidly across bilayers that contain cardiolipin, possibly including the inner mitochondrial membrane.

Intrinsic Proteins Intrinsic or integral membrane proteins cannot be

solubilized without detergent and contain one or more hydrophobic sequences which span the lipid bilayer one or more times in α -helical structures. Studies on the interactions of lipids with such proteins have resulted in a particularly large literature. This work has mainly focused on the specificity of such lipid-protein interactions and on the physical state of **the** lipid (Jost and Griffith 1982). In particular, it has been shown that lipids residing at the lipid-protein interface of intrinsic proteins experience a different environment than do bulk bilayer lipids. It has been speculated that such boundary lipids may be specific to a given protein and **provide environments** that are appropriate to, and that possibly regulate, function. These theories were supported by early ESR studies of spin-labeled lipids in reconstituted systems which demonstrated that such lipids, when in the vicinity of integral proteins, exhibited increased order parameters (that is, restricted motion of the lipid) in the acyl chain region. Other studies indicating the importance of the physical state of boundary lipids demonstrated that gel-state boundary lipids inhibited the function of the sarcoplasmic reticulum Ca^{2+} ATPase and other membrane-bound enzymes in reconstituted systems.

More recent work is pointing to a rather different picture, however.

First, with the exception of a possible requirement for one **two** molecules of a particular lipid, lipid-protein interactions appear relatively nonspecific, in that a large variety of different (liquid-crystalline) lipids can usually support protein activity. The sarcoplasmic reticulum ATPase, for example, has excellent activity when reconstituted with a variety of phospholipids as well as detergents (Dean and **Tanford** 1977). Similar observations have been made for many other integral proteins, including cytochrome **oxidase**. A second point is that, in general, a long-lived boundary layer of lipid does not appear to exist at the lipid-protein interface. For example, whereas ESR spin-label studies indicate long-lived boundary **compo-**

nents, ^2H -NMR studies *on analogous* systems containing ^1H -labeled lipids do not reveal such components. This apparent discrepancy has been reconciled, since ESR and NMR report on phenomena occurring during different time scales. Boundary-bulk lipid exchange rates in the region 10^{-6} – 10^{-8} s would appear slow on the ESR time scale but fast on the NMR time scale. These observations, together with NMR and calorimetric results indicating that integral proteins can have disordering effects on adjacent lipids, suggest that lipids in the region of intrinsic protein are in relatively rapid exchange ($T_{ex} \sim 10^{-7}$ s) and do not have gel-state characteristics. This does not mean that the lipid composition in contact with the protein is necessarily the same as the bulk composition, as effects such as electrostatic lipid-protein interactions may enhance the local concentration of a particular lipid species on a time-averaged basis. Furthermore, such generalizations may not hold for particular situations. The purple membrane fragments of *Halobacterium halobium*, which contain bacteriorhodopsin, for example, exhibit a unique lipid composition distinct from the rest of the membrane (Stoekenius 1976).

The influence of intrinsic proteins on lipid polymorphism has received less detailed attention; however, some interesting features are emerging (de Kruijff et al. 1984). First, the hydrophobic **peptide** antibiotic gramicidin, which spans the membrane as a dimer, has a very strong bilayer destabilizing capacity and even induces H_{II} phase structure in phosphatidylcholine systems. On the other hand, *glycophorin*, the major asialoglycoprotein from the erythrocyte, stabilizes the bilayer structure for unsaturated phosphatidylethanolamines. Thus, the message from these initial studies is clear—membrane proteins may well play an active role in determining local membrane structure.

In summary, our understanding of lipid-protein interactions in biological membranes remains relatively unsophisticated. It may be that some fraction of lipid diversity satisfies relatively nonspecific requirements and provides an appropriate solvent for the optimal function of integral proteins. Alternatively, specific functions of lipids may be more related to other membrane properties, such as permeability, than to protein function per se. *In addition*, many **fundamental** questions have not yet been adequately addressed, including the role of various lipids in insertion of protein, in sealing proteins within the bilayer matrix, and in providing an interface appropriate for membrane protein-substrate interactions.

LIPIDS AND MEMBRANE FUSION

Membrane fusion (in various expressions) is one of the most ubiquitous membrane-mediated events, occurring in processes of fertilization, cell division, exo- and endocytosis, infection by **membrane-bound** viruses, and intracellular membrane transport, to name but a few.

There are strong experimental and theoretical indications that the lipid components of membranes are directly involved in such fusion processes. For example, **model** membrane systems such as **LUVs** can be induced to fuse in the absence of any protein factors. In addition, it is **topologically** impossible for two membrane-bound systems to fuse together to achieve mixing of internal compartments without a local transitory departure from the normal lipid bilayer structure at the fusion interface. We shall discuss the possible nature of the fusion intermediates, as indicated by studies on **model** membrane systems. The fusion intermediates are subsequently related to fusion behavior observed in biological membrane systems.

Fusion of Model Systems

For fusion events to proceed *in vivo* the presence of Ca^{2+} is often required. As a result, numerous studies have been concerned with the induction of **Ca^{2+} -stimulated** fusion between vesicle systems and analysis of the lipid factors involved. We shall discuss in turn the modulation of gel-liquid-crystalline properties of lipids and the modulation of the **polymorphic** properties of lipids in relation to membrane fusion.

It has been recognized for some time that model membrane SUV systems will undergo fusion when incubated at temperatures in the region of their gel-liquid-crystalline transition temperature T_c . Continued recycling of sonicated dipalmitoylphosphatidylcholine vesicles through $T_c = 41^\circ\text{C}$, for example, results in fusion and formation of larger systems. Isothermal induction of crystalline structure by the addition of Ca^{2+} to phosphatidylserine systems results in fusion to form the large crystalline **cochleate** structures noted previously (Papa-hadjopoulos, Poste, and Vail 1978). Given the involvement of Ca^{2+} in biological fusion events, the latter observation suggests that Ca^{2+} may induce lateral segregation of negatively charged phospholipids, such as phosphatidylserine, *in vivo*, which may act as local crystalline nucleation points for fusion. However, phosphatidylserine is not always present in membranes which undergo fusion, nor is Ca^{2+} able to induce crystalline cochleate-type structures for other species of (unsaturated) negatively charged phospholipids. Furthermore, in more complex lipid mixtures containing phosphatidylethanolamine and cholesterol, for example, there are strong indications that Ca^{2+} is not able to induce segregation of unsaturated **phosphatidylserines** (Tilcock et al. 1984). Finally, the concentration of Ca^{2+} required to induce crystalline **phosphatidylserine- Ca^{2+}** complexes is **2mM** or larger, a concentration much higher than could occur in the, cell **cytoplasm, for example**.

The hypothesis that membrane fusion proceeds by taking advantage of the polymorphic capabilities of component lipids is more

viable, but not proven. Three important observations have been made which support this hypothesis. First, it has been shown that lipid-soluble *fusogens* (such as glycerolmonooleate, which induces cell fusion in vitro), induce H_{II} phase structures in model and biological membranes (Cullis et al. 1983), which is consistent with a role of nonbilayer structure during fusion. Second, MLV systems composed of lipid mixtures such as phosphatidylethanolamine and phosphatidylserine form H_{II} structures on the addition of Ca^{2+} . SUV or LUV systems with this lipid composition first fuse to form larger lamellar systems exhibiting lipidic particle structures (as shown in Figures 2.11 and 2.14), before assuming the H_{II} arrangement. Finally, a variety of factors which engender H_{II} organization, such as pH variation or increased temperatures, can induce fusion of vesicle systems with appropriate lipid compositions (Tilcock et al. 1982; Verkeleij 1984; de Kruijff et al. 1984).

These observations have led to a general hypothesis that factors which tend to induce nonbilayer (H_{II} phase) structure will also induce fusion between membrane-bound systems. There are many attractive features to this hypothesis. In particular, lipids which adopt H_{II}

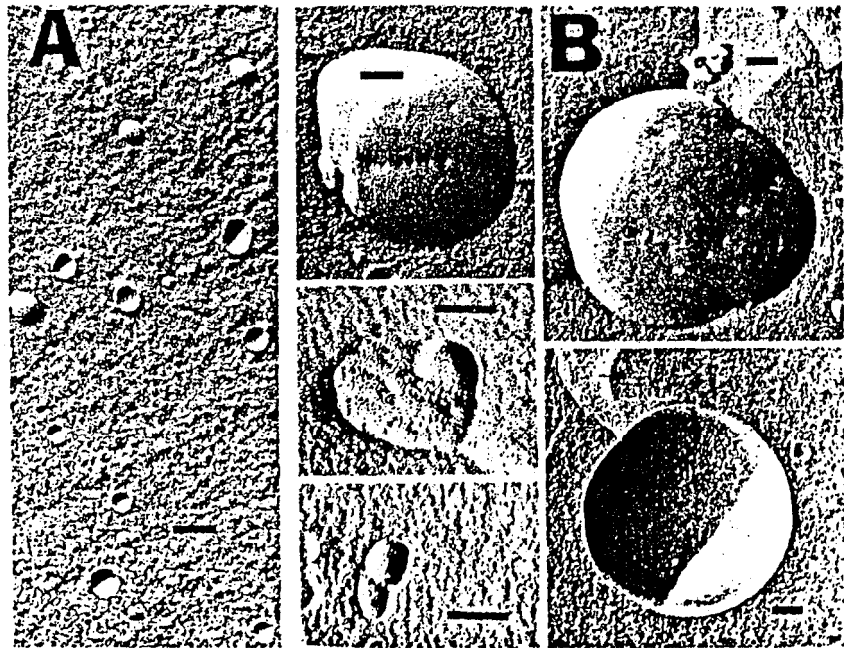


Figure 2.24. (A) Freeze-fracture micrographs of vesicles composed of soy phosphatidylethanolamine containing 20 mol % soy phosphatidylserine.

(B) The vesicles undergo fusion to large bilayer structures containing lipidic particles following the addition of $2mM Ca^{2+}$ at room temperature. Each bar represents 100nm.

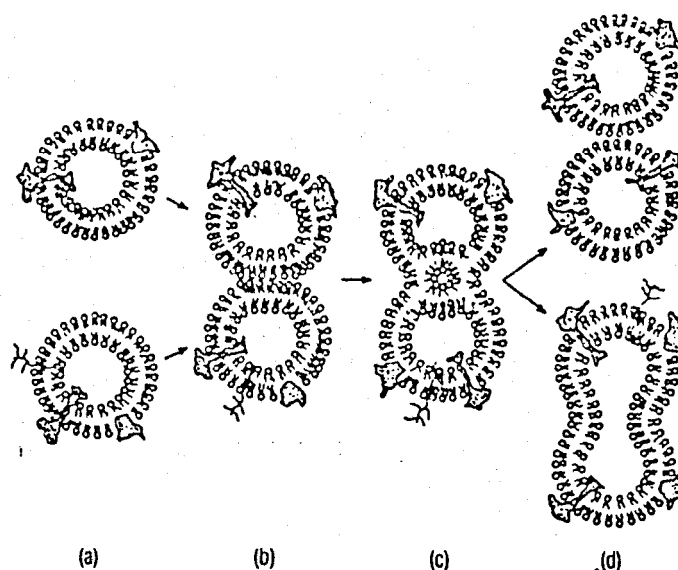


Figure 2.15. **Proposed** mechanism of membrane fusion proceeding via an inverted cylinder or inverted micellar intermediate. The process whereby the membranes come into close apposition (a)-(b) is possibly protein mediated, whereas the fusion event itself (b)-(c) is proposed to involve formation of an inverted lipid intermediate.

organization hydrate poorly in comparison with bilayer lipids and thus allow the close apposition of membranes required for fusion. In addition, the ability of such lipids to adopt inverted structures, such as inverted micelles or inverted cylinders, clearly provides an attractive intermediate structure for fusion. Furthermore, all membranes appear to contain lipids that can adopt nonbilayer structures, and a large number of biologically relevant variables can modulate the structural preferences of these lipids. These facts support the proposition that fusion proceeds via a nonbilayer intermediate, as shown in Figure 2.15. Difficulties with this model are that lipidic particle structures observed on fusion (Figure 2.14) appear to be generated subsequent to the actual fusion event and that for **Ca²⁺-induced** fusion, high Ca²⁺ levels (greater than **0.5mM**) are required. However, the nonbilayer fusion intermediates may be extremely short-lived and thus difficult to observe by freeze-fracture. Other factors, such as **Mg²⁺** or protein, may act synergistically with **Ca²⁺** to induce fusion *in vivo* at lower **Ca²⁺** levels.

Fusion of Biological Membranes Extension of the preceding observations on fusion of model systems to fusion processes *in vivo* is difficult to show directly. However, work on several experimental systems has provided circumstantial

evidence in support of the hypothesis that fusion processes rely on the polymorphic capabilities of lipids. One system studied was the fusion process involved in the exocytotic events occurring during release of the contents of secretory vesicles such as the chromaffin granules of the adrenal medulla (Cullis et al. 1983). Such exocytosis is dependent on the influx of Ca^{2+} , which stimulates fusion between the granule and the cytosolic side of the plasma membrane. By analogy to the erythrocyte membrane, the inner (cytosolic) monolayer is probably composed primarily of phosphatidylserine and phosphatidylethanolamine. Studies have shown that chromaffin granules will undergo Ca^{2+} -stimulated fusion with SUVs of inner-monolayer lipid composition. Such fusion appears to depend on the ability of Ca^{2+} to promote nonbilayer structures. In another system, myoblast cells (which fuse to form the multinucleated muscle fibers) have been studied (Sessions and Horwitz 1981). Such fusion, which is also Ca^{2+} dependent, may rely on a different transmembrane distribution of phosphatidylethanolamine and phosphatidylserine, which appear to reside mainly in the outer monolayer of the myoblast plasma membrane.

Yet another system concerns the tight junction network formed by epithelial and endothelial cells to separate apical (membrane facing the lumen) and basolateral (surface opposite the lumen) domains. Such networks may correspond to a situation of arrested fusion. Recent freeze-fracture work suggests that the striated patterns characteristic of tight junction assemblies (Figure 2.16) may correspond to long, inverted lipid cylinders similar to those comprising the H_{II} phase structure (Kachar and Reese 1982). Similar states of arrested

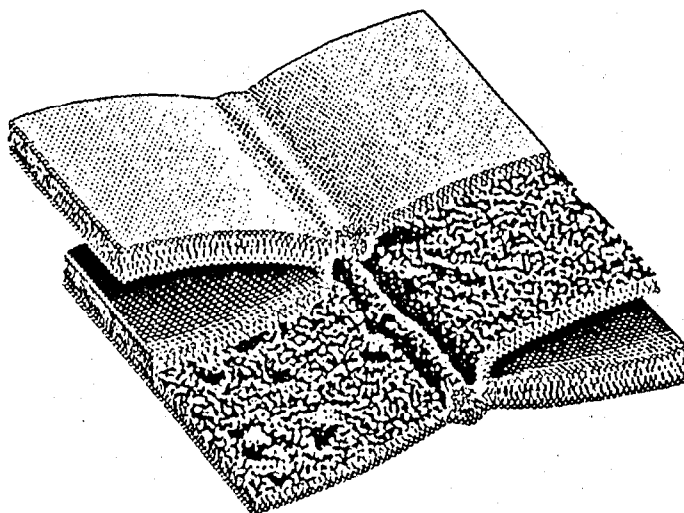


Figure 2.16. Diagram of a cross section of a tight junction strand combined with freeze-fracture micrographs. Reproduced with permission from Kachar and Reese (1982).

fusion may correspond to the contact sites between the inner and outer membranes of mitochondria and *E. coli*.

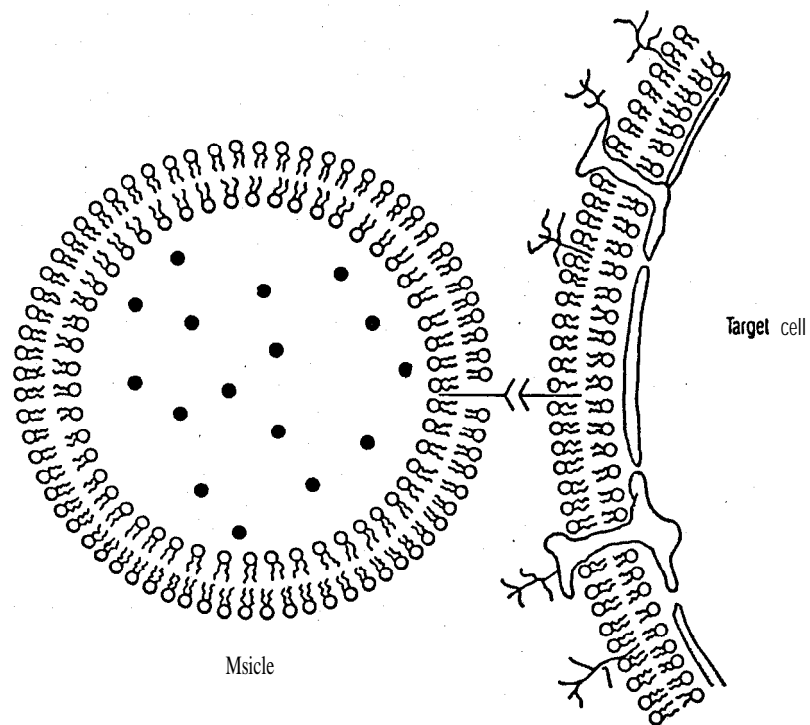
MODEL
MEMBRANES AND
DRUG DELIVERY

The preceding sections have dealt primarily with the use of lipids in various model membrane systems to gain insight into the physical properties and relative functional roles of individual lipid components in biological membranes. However, these model membrane systems have important potential uses in their own right, as carriers of biologically active agents such as drugs, enzymes, and DNA vectors for clinical application (Poste 1980). Natural membrane lipid components such as phosphatidylcholine are remarkably nontoxic and nonimmunogenic and can therefore provide benign carriers for more toxic or labile agents encapsulated within lipid vesicles. An important aim, which has not yet been realized, is to target liposomal systems containing drugs such as anticancer agents to specific tissues via antibodies attached to the vesicle surface, as indicated in Figure 2.17.

The many, difficulties involved in drug delivery via liposomal systems may be summarized as follows: First, vesicle systems must be employed which exhibit an adequate trapped volume to entrap sufficient drug, and a mode of preparation must be used which allows a high trapping efficiency. Several such procedures now exist, including the reversed-phase evaporation protocol (Szoka and Papahadjopoulos 1980) and the extrusion protocol (Hope et al. 1985) outlined previously (Table 2.4), which allow maximum trapping efficiencies in the range of 30–50% of available drug. The second difficulty concerns the phenomenon of serum-induced leakage of the liposomes due to interaction with serum components such as lipoproteins. This problem can be significantly alleviated by inclusion of lipids that are more saturated and/or cholesterol in the carrier vesicle. A third and major difficulty for liposomal delivery systems involves uptake of the liposomes by the fixed and free macrophages of the reticuloendothelial system, which are primarily localized to the liver (Kupffer cells) and spleen. This problem has not yet been circumvented, and even if such uptake could be avoided, other significant problems would remain. For instance, although several procedures exist for coupling antibodies to vesicles, it is unlikely that such targeted systems will be able to cross the endothelial barrier to gain access to extravascular tissue.

Despite these problems, the attractive nature of vesicle-mediated drug delivery has engendered increasing interest and effort which have already resulted in protocols of potential clinical importance. These advances have taken advantage of the natural targeting to macrophages, with two distinct aims. The first involves parasites which reside in the macrophages and which are difficult to eliminate

Figure 2.17. The delivery of biologically active materials encapsulated in membrane vesicles. Tissue-specific antibodies are covalently attached to the surface of the vesicle and enable the targeting of entrapped material.



by conventional means. However, encapsulation of an appropriate drug into a vesicle carrier which is subsequently taken up by the macrophages can result in elimination of parasites such as *Lieshmania* (Alving 1982). An advantage of this method of treatment is that the dose levels needed are at least an order of magnitude lower than otherwise required. A second clinical application of vesicle-mediated drug delivery is the incorporation of macrophage-activating factors into the vesicles which, when endocytosed, result in **macrophage** activation. Such activated macrophages appear to be remarkably effective for recognizing and destroying diseased tissue, including transformed cells (Poste 1980).

PROBLEMS

1. The phospholipids of the erythrocyte membrane are asymmetrically distributed across the **mem-**brane. On the basis of the lipid composition of the two monolayers, which monolayer would you expect to be the most permeable to Na^+ or to Cl^- ions? How would you verify this experimentally?
2. The apical and **basolateral** domains of polarized epithelial and endothelial cells exhibit different lipid and protein compositions. It has been observed that lipids introduced 'into the outer monolayer of the apical region remain there, whereas lipids which can redistribute across the **bilayer** are subsequently found in both apical and

- basolateral domains. Indicate how the barrier presented by the arrested fusion model of **Figure 2.16 is compatible** with these properties.
- The microorganism *Acholeplasma laidlawii* can be manipulated so that its fatty acid composition is essentially homogeneous. It is observed that as the acyl chain **unsaturation** is increased, the ratio of endogenous **monoglucosyldiglyceride (MGluDG) to diglucosyldiglyceride (DGluDG)** decreases dramatically. MGluDG is an H_{II} phase lipid, whereas DGluDG is a bilayer lipid. Indicate how the change in ratios of MGluDG to DGluDG could be rationalized in terms of lipid shape properties.
 - Valinomycin is a relatively specific K^+ ionophore, which **translocates K^+ ions** across membranes. LUV systems are prepared so that a K^+ buffer is on the inside and a Na^+ buffer is on the outside. How would you expect the efflux of the K^+ ions to vary with time when valinomycin is added? How would you measure this?
 - Indicate four procedures which you would expect to induce fusion between LUVs composed of a mixture of unsaturated phosphatidylserine and phosphatidylethanolamine.

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