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CHAPTER 1

Physical properties and functional roles of lipids in membranes

PIETER R. CULLIS[1,2], DAVID B. FENSKE and MICHAEL J. HOPE[2,3]

[1]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, B.C., V6T 1Z3, Canada, [2]Inex Pharmaceuticals Corp., 1779 W. 75th Avenue, Vancouver, B.C., V6P 6P2, Canada and [3]Division of Dermatology, Faculty of Medicine, University of British Columbia, Vancouver, B.C., V5Z IL7, Canada

1. 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 [l], who extracted lipids from the erythrocyte membrane and measured the areas these lipids were able to cover as a monolayer at an air-water interface. This work led to the 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, 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 Nicholson [2] fluid mosaic model, a refined version of which is shown in Fig.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 polar or hydrophilic (water loving) head group region and non-polar 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 towards the aqueous phase, while the hydrophobic sections are sequestered from water. In addition to the familiar bilayer phase, a number of other

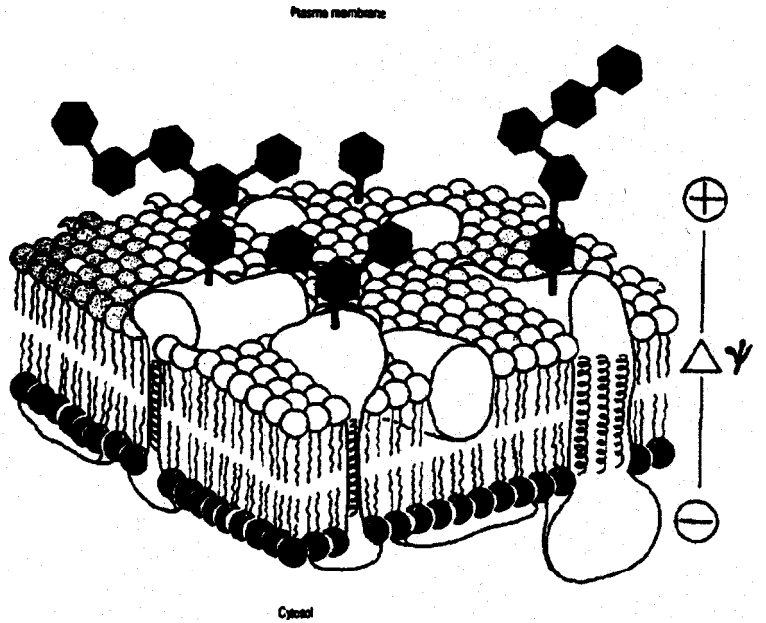


Fig. 1. The topography of membrane protein, lipid and carbohydrate in the fluid mosaic model of a typical eukaryotic plasma membrane. Phospholipid asymmetry results in the preferential location of PE and PS in the cytosolic monolayer. Carbohydrate moieties on lipids and proteins face the extracellular space. $\Delta\psi$ represents the transmembrane potential, negative inside the cell.

macromolecular structures are compatible with these constraints. It is of particular interest that many naturally occurring lipids prefer non-bilayer 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 usually all, membrane lipids are fluid; thus, the major emphasis of this chapter concerns 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 non-specific leakage is prevented and an environment appropriate to a functional protein conformation is provided.

More extended discussions of biomembranes and the roles of lipids can be found in the excellent text by Gennis [3].

2. *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 discuss the diverse chemistry of the sub-class of lipids which are found in membranes. This excludes other lipids which are poorly soluble in bilayer membrane systems, such as triacylglycerols and cholesteryl esters.

2.1. *Chemical diversity of lipids*

The major classes of lipids found in biological membranes are summarized in Fig. 2. In eukaryotic membranes the glycerol-based phospholipids are predominant, including phosphatidylcholine PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and cardiolipin. Sphingosine-based lipids, including sphingomyelin 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 eukaryotes (Chapter 12). Cholesterol is also a major component of eukaryotic membranes, particularly in mammalian plasma membranes, where it may be present in equimolar proportions with phospholipid.

In most prokaryotic membranes, PC is not usually present (Chapter 2), whereas the major phospholipids observed are PE, phosphatidylglycerol, and cardiolipin. In plant membranes on the other hand, lipids such as monogalactosyl and digalactosyl diacylglycerols can form the majority components of membranes such as the chloroplast membrane (Chapter 14).

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, phosphatidylinositols, 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 l-position of the glycerol backbone and an unsaturated fatty acid at the 2-position. Also, in eukaryotic membranes it is usual to find that PE and PS, 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 I the fatty acid composition of phospholipids found in the human erythrocyte membrane. From this table it is clear that the number of molecular species of phospholipids in a membrane can easily exceed 100.

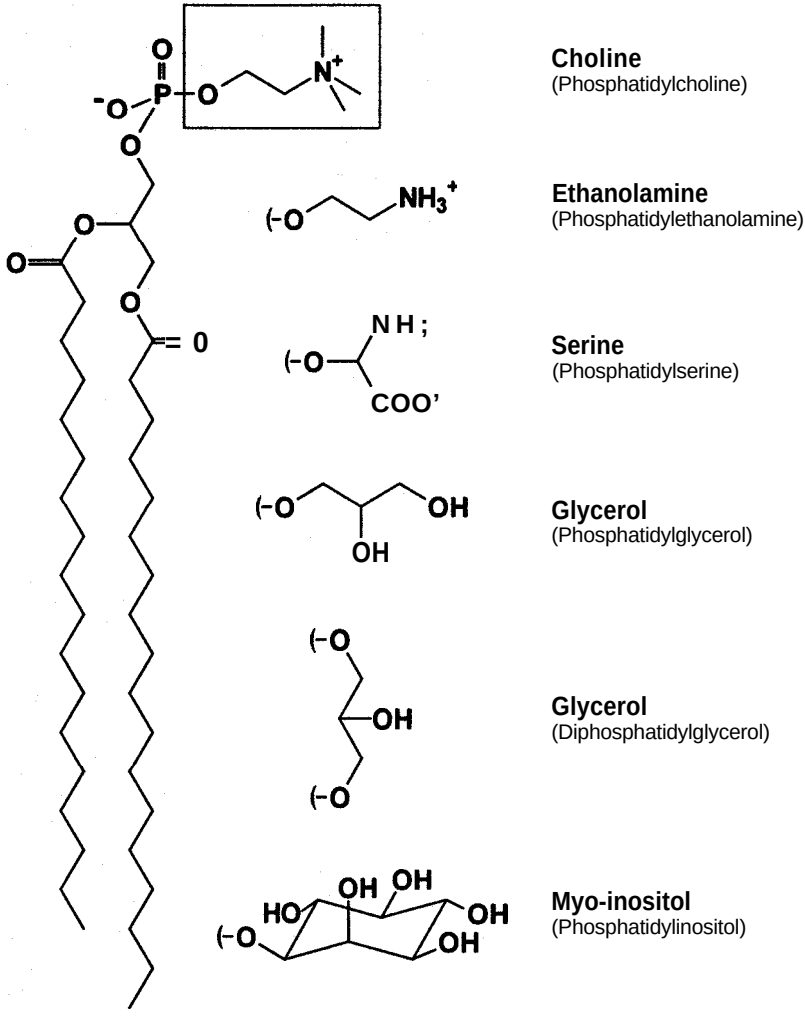


Fig. 2. The structure of the phospholipid molecule distearoyl-PC in the liquid crystalline state is represented schematically. Head groups for the other classes of phospholipid are also shown. The glycerol moiety of cardiolipin (diphosphatidylglycerol) is esterified to two phosphatidic acid molecules.

2.2. *Membrane lipid compositions*

The lipid compositions of several mammalian membrane systems are given in Table II (see also Chapter 15). Dramatic differences are observed for the cholesterol contents. Plasma membranes such as those of myelin or the erythrocyte contain approximately equimolar quantities of cholesterol and phospholipid, whereas the organelle membranes of endoplasmic reticulum or the inner mitochondrial membrane contain only small

TABLE I

Gas chromatographic analyses of the fatty acid chains in human red cell phospholipid

Chain length and unsaturation	Total phospholipids	SPM	PC	PE	PS
16:0[a]	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:o	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

The data am expressed as weight% of the total. SPM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. + denotes that the concentration did not exceed 1% of the total. Reproduced with permission of Van Deenen and de Gier (1974) Lipids of the red cell membrane, in: D. Surgenor (Ed.), The Red Blood Cell, Academic Press, New York, pp. 147-213.

[a]This code indicates the number of carbon atoms in the chain and the number of double bonds.

amounts of cholesterol. This cholesterol distribution correlates well with the distribution of sphingomyelin. Cholesterol may have a 'fluidizing' role in membranes containing sphingomyelin, which is relatively saturated. Cardiolipin is almost exclusively localized

TABLE II

The lipid composition of various biological membranes

Lipid	Erythrocyte[a]	Myelin[a]	Mitochondria[b] (inner and outer membrane)	Endoplasmic reticulum[b]
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

The data are expressed as weight % of total lipid.

[a]Human sources.

[b]Rat liver.

TABLE III

Double-bond composition of phospholipids of various membranes

Myelin	**0.5[a]**
Erythrocyte	**1.0**
Sarcoplasmic reticulum	**1.4**
Mitochondria (inner membrane)	**1.5**
Nerve synapse	**>2**

[a]Average number of double bonds per phospbolipid molecule.

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 III.

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

2.3. *Transbilayer lipid asymmetry*

The inner and outer leaflets of membrane bilayers may exhibit different lipid compositions [4]. The plasma membrane of human erythrocytes is 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 PE 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 PE can be detected at the outer surface, with 80% confined to the inner monolayer. The outer monolayer consists predominantly of PC, sphingomyelin, and glycolipids. Figure 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 information available for organelle membranes suggests that PE and PS are also oriented towards the cytosol. A general feature of plasma membrane asymmetry is that the majority of phospholipids that exhibit a net negative charge at physiological pH (PS and PI; PE is only weakly anionic) are limited to the cytosolic half of the bilayer. Certain proteins appear to be involved in maintaining this asymmetry (Chapter 15). Treatment of erythrocytes with diamide, which induces cross-linking of the cytoskeletal protein spectrin, results in the appearance of PS in the outer monolayer. Red blood cells known to have lesions associated with cytoskeletal proteins also exhibit a partial breakdown of asymme-

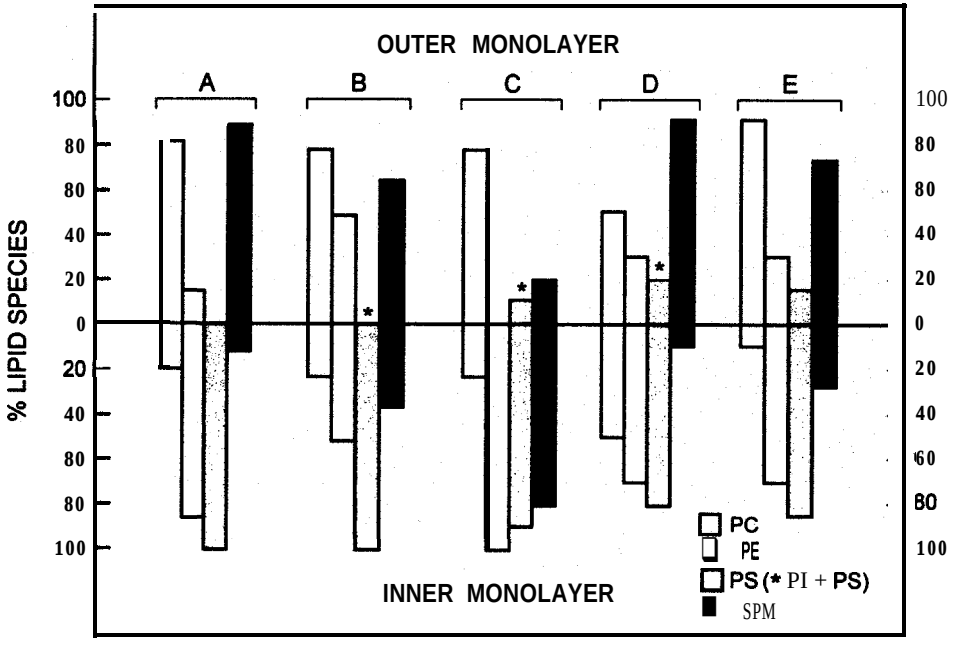


Fig. 3. Phospholipid asymmetry in plasma membranes. (A) Human erythrocyte membrane, (B) rat liver blood sinusoidal plasma membrane, (C) rat liver plasma membrane, (D) pig platelet plasma membrane, (E) VSV envelope derived from hamster kidney BHK-21 cells.

try, with an increased exposure of PS and PE on the outer half of the bilayer and an equivalent transfer of PC to the inner monolayer.

These experiments suggest a possible interaction between cytoskeletal proteins and membrane phospholipids to generate and maintain asymmetry. However, some phospholipids will redistribute across the bilayer of protein-free model membrane systems in response to transmembrane pH gradients. Phosphatidylglycerol and phosphatidic acid, for example, will diffuse to the inner monolayer of large unilamellar vesicles that exhibit an interior pH that is basic with respect to the external pH [5]. Similar responses to transmembrane proton gradients would be expected to occur in vivo. On the other hand, an aminophospholipid translocase (see also Chapter 15) has been identified in a number of plasma membranes which appears to be responsible for the movement of PE and PS across the bilayer 141. This ATP-dependent 'lipid pump' activity has also been found in organelle membranes but oriented such that the aminophospholipids are transported from the inner monolayer to the outer monolayer, which is consistent with their phospholipid asymmetry.

The functional importance of lipid asymmetry is not clear but could be related to prevention of exposure of PS at the outer surface of a normal cell, which has been suggested to be a signal of senescence [6]. Alternatively, PE and PS may be required to maintain a fusion competent surface for endocytosis and organelle fusion (see Fig. 10 and [4]).

3. 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.

3. I. *Lipid isolation and purification*

Although a wide range of synthetic and natural lipids are now commercially available, a variety of techniques have been developed for isolation of lipids from membranes [7]. In the preparation of erythrocyte phospholipids, the first step involves disruption of the membrane in a solvent 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 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, small scale isolation, 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.

High-pressure liquid chromatography enables the rapid purification of large quantities of natural lipids. Analytical high pressure liquid chromatography techniques are well-developed for the rapid separation of phospholipids by headgroup and acyl chain composition. Reversed-phase chromatography, where the stationary phase is hydrophobic and the mobile phase hydrophilic, is particularly useful. 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 and degree of unsaturation.

3.2. Techniques for making model membrane vesicles

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 separated by narrow aqueous spaces. 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 motional 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 [8].

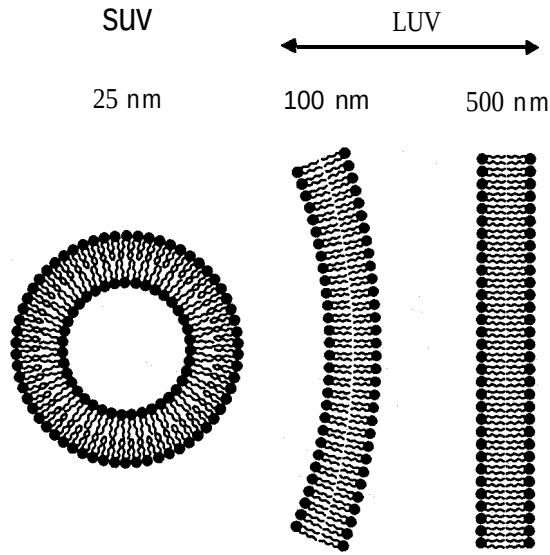
Small unilamellar vesicles (SUVs) can be made from MLVs by subjecting the MLVs to ultrasonic irradiation or by passage through a French press. However, their small size limits their use in model membrane studies. Typically, diameters in the range 25-40 nm are observed. The radius of curvature experienced by the bilayer in SUVs is so small (Fig. 4) 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 which 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 (LW) system, where the mean diameter is larger, and the distribution of lipid between the outer and inner monolayers is closer to 1:l. The most common procedures for producing LUVs result in unilamellar vesicles with diameters ranging from 50 to 500 nm. These preparative procedures often include the use of detergents or organic solvents, although LUVs can be produced directly from MLVs.

The most popular technique for making LUVs involves the direct extrusion of MLVs under moderate pressures (1500 psi) through polycarbonate filters of defined pore size. This process can generate LUVs with size distributions in the range of 50-200 nm depending on the pore size of the filter employed [8]. Extrusion does not require detergents or solvents, which are difficult to remove, and it can be applied to all lipids which adopt liquid crystalline bilayer structures, including long chain saturated lipids. The technique is rapid, straightforward and convenient, allowingLUVs to be prepared in 10 min or less.

3.3. *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. 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 sol-



Diameter (nm)	IM/OM (mole ratio)	Trap (µl per µmol)	No. phospholipid molecules per vesicle	No. vesicles per µmol of lipid
25	0.46	0.3	4.6×10^3	1.3×10^{14}
100	0.65	2.5	9.7×10^4	6.2×10^{12}
500	0.97	15	2.6×10^6	2.3×10^{11}

Fig. 4. The cwature and some characteristics of large unilamellar vesicles (LUV) and small unilamellar vesicles *(SW)*. LUVs typically have diameters in the range 100-500 nm. SUVs prepared by sonication can be as small as 25 nm in diameter. The radius of curvature for each vesicle sire is shown in proportion. The ratio of lipid in the inner monolayer (lM) 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.

vent collects at the **perimeter** of the aperture, leaving a bilayer film across the center. The electrical properties of the barrier are readily measured by 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. More recent techniques avoid some of these problems [9].

Another planar bilayer model is the oriented multibilayer, which consists of membrane lamellae sandwiched between glass plates. These models are primarily utilized in biophysical NMR studies of membrane lipid structure and motion. The lipid mixture of choice is dissolved in an organic solvent and streaked onto glass plates, which are then stacked and placed under high vacuum to remove residual solvent. Hydration of the lipid and formation of the multibilayers occurs during a 24 h incubation in a humid atmosphere.

In 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 [l0].

3.4. *Reconstitution of integral membrane proteins 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 [ll]. 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 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, then removing the detergent by dialysis. 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 [1 **1].** Finally, the systems should have asymmetric protein orientation. In contrast with the intact biological membrane (Chapter **16),** 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

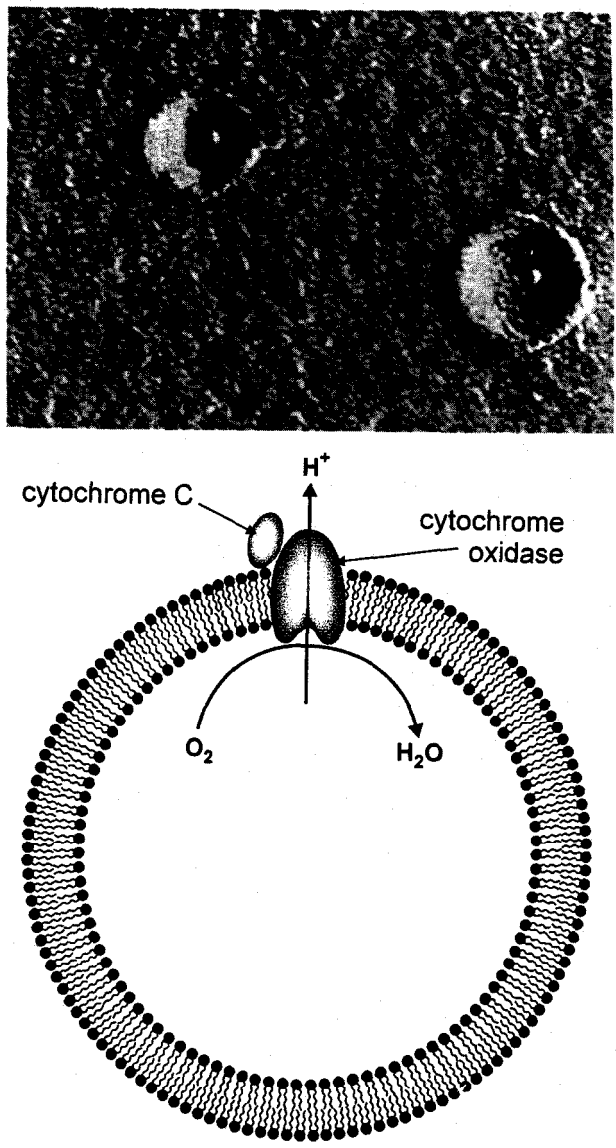


Fig. 5. Unidirectionally shadowed freeze-fracture micrographs of cytochrome c oxidase reconstituted with dioleoyl-PC. The protein to lipid ratio is <l:5000 (w/w). The vesicle diameter is approximately 100 nm. Each particle represents one dimer of cytochrome c oxidase and is approximately 10 nm in diameter [T.D. Madden, 1988]. The orientation of the reconstituted protein is shown in the diagram below.

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 Fig. 5.

In some cases asymmetric incorporation of other proteins can be achieved by different procedures. Erythrocyte glycophorin, 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 glycophorin, 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 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 20 mM) which are sufficient to solubilize the spike protein of Semliki Forest virus [12]. 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 domain of the molecule can insert into the preformed bilayer upon dialysis.

In summary, a large variety of sophisticated and well-defined model membrane systems are available. The 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.

4. *Physical properties of lipids*

4.1. *Gel-liquid-crystalline phase behavior*

As indicated previously, membrane lipids can exist in a frozen gel state or fluid liquid-crystalline state, depending on the temperature [13], as illustrated in Fig. 6. Transitions between the gel and liquid-crystalline phases can be monitored by a variety of techniques, including nuclear magnetic resonance (NMR), electron spin resonance, and fluorescence. Differential scanning calorimetry, which measures the heat absorbed (or released) by a sample as it undergoes an endothermic (or exothermic) phase transition, is particularly useful. A representative scan of dipalmitoyl-PC, which exhibits a gel to liquid-crystalline transition temperature (T_c) of 41°C is illustrated in Fig. 6. 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; and 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.

It is worth emphasizing two general points. First, gel-state lipids always assume an overall bilayer organization, presumably because the interactions between the crystalline acyl chains are maximized. Thus, the non-bilayer 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 non-cooperative 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.

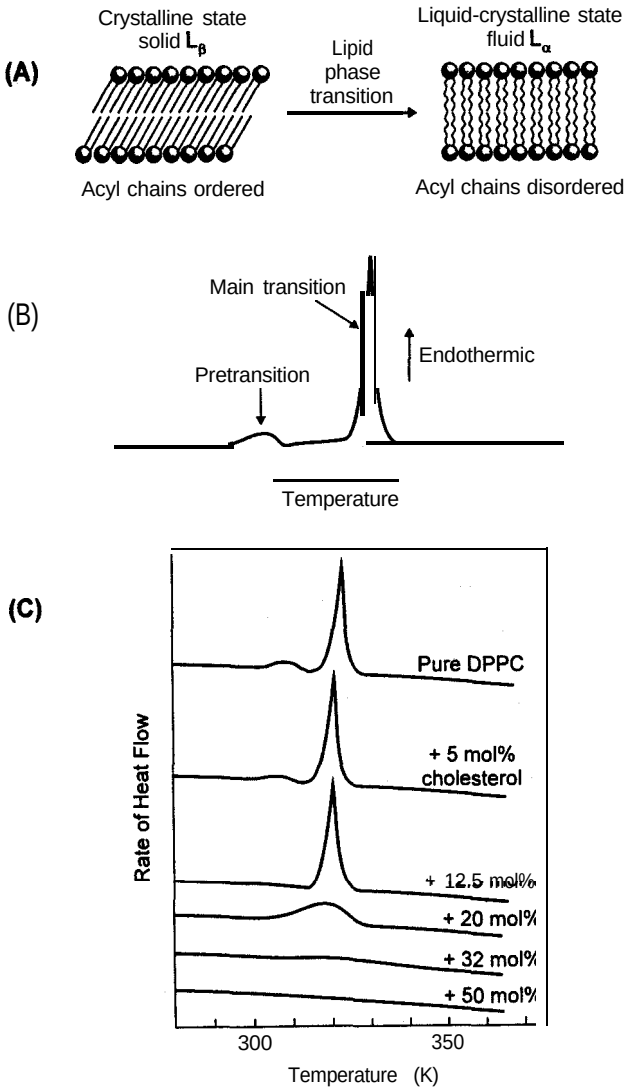


Fig. 6. The phosphoiipid gel-liquid-crystalline phase transition and the effect of cholesterol. (A) Phospholipids, when fully hydrated, can exist in the gel, crystalline form (L_β) or in the fluid, liquid-crystalline state (L_α). In bilayers of gel-state PC, the molecules can be packed such that the acyl chains are tilted with respect to the bilayer normal ($L_{\beta'}$ state). (B) Raising the temperature converts the crystalline state into the liquid-crystalline phase as detected by differential scanning calorimetry. For dipalmitoyl-PC 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.

TABLE IV

Temperature (T_c) and enthalpy (AH) of the gel to liquid-crystalline phase transition of phospholipids (in excess water)

Lipid species[a]		$T_c \pm 2°C$	AH ± 1 kcal/mol
12:0/12:0	PC[b]	-1	3
14:0/14:0	PC	23	6
16:0/16:0	PC	41	8
16:0/18:1cΔ^9	PC	-5	
16:1cΔ^9/16:1cΔ^9	PC	-36	9
18:0/18:0	PC	54	
18:1cΔ^9/18:1cΔ^9	PC	-20	9
16:0/16:0	PE	63	9
16:0/16:0	PS	55	9
16:0/16:0	PG	41	9
16:0/16:0	PA	67	5

[a]The code denotes the number of carbons per acyl chain and the number of double bonds. A gives the position of the double bond, c denotes *cis*.

[b]PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid.

The calorimetric behavior of a variety of synthetic phospholipids is given in Table IV. There are three points of interest. First, for the representative phospholipid species, PC, 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. Inclusion of only one cis-unsaturated fatty acid at the *sn*-1 or *sn*-2 position of the glycerol backbone is sufficient to lower T_c from 41°C for dipalmitoyl-PC to –5°C for the palmitoyl-oleoyl species, a major molecular subspecies of PC 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 PE commonly exhibit T_c values 20°C higher than corresponding species of PC. The data of Table IV have some predictive value in that approximate values of T_c can be estimated for other molecular species of lipids.

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 PCs differing by four carbon units or more (AT, > 20°C) can exhibit lateral phase separation (indicated by calorimetric and freeze-fracture studies).

Further studies of the calorimetric behavior of lipid systems have emphasized the remarkable physical properties of cholesterol [10,14]. This lipid has the ability to inhibit the crystallization of lipids to form gel-state systems, as illustrated for dipalmitoyl-PC in Fig. 6C. The enthalpy of the transition is progressively reduced as the cholesterol content is increased, until for PC:cholesterol molar ratios of 2:1 and less, no transition is observable. Such mixtures exist in the 'liquid-ordered' phase, in which the membranes are 'fluid' but highly ordered (as characterized by ^{2}H-NMR and DSC measurements) [14].

Gel-liquid crystalline transitions profoundly influence the motional properties of lipids and therefore are readily detected by NMR techniques. In the liquid crystalline phase, lipids can rotate rapidly about their long axis and diffuse rapidly in the plane of the bilayer. In the gel phase, such motions are inhibited. ^{2}H-NMR is of particular utility for characterizing both structure and motion in the hydrocarbon region of liquid crystalline bilayers. The extent of molecular motion of any C–^{2}H bond can be quantified by an order parameter S, derived from the width of ^{2}H-NMR spectra, where $S = 1$ indicates a fully ordered system and S = 0 indicates isotropic (completely disordered) motion where the ^{2}H nucleus is able to assume all possible orientations with respect to the magnetic field within $\sim 10^{-6}$ s. A plot of the order parameter values for each position of an acyl chain, referred to as an order profile, can be generated employing phospholipids labeled specifically in the acyl chain region or, more conveniently, by employing phospholipids containing perdeuterated fatty acids [14]. Hydrocarbon regions of bilayer systems exhibit a characteristic order profile with a 'plateau' region near the headgroup, after which the order decreases rapidly towards the center of the bilayer [14]. Hydrocarbon order can be modulated by a variety of factors such as cholesterol or increased acyl chain saturation, both of which lead to larger order parameters.

The relation between the gel-liquid crystalline properties of lipids and the roles of lipids in biological membranes remains obscure. Suggestions that particular lipids may segregate into gel domains within a biological membrane, with possible effects on protein function (due to restricted mobility) or membrane permeability (due to packing defects), suffer in two respects. First, there is simply no evidence for the presence of gel-state lipid components at physiological temperatures in eukaryotic membranes, although this has been suggested for certain prokaryotic systems. Second, there is no obvious mechanism whereby lateral segregation of lipid into crystalline domains might be regulated. Clearly, an organism cannot regulate fluidity by regulating temperature; thus, any such mechanism would require physiological factors that would isothermally modulate the local lipid composition. The presence of such factors has not been unambiguously demonstrated.

The theme that membranes do not require the presence of gel-state lipids is easily developed for eukaryotic membrane systems, such as the well-characterized erythrocyte membrane. Of the erythrocyte membrane lipids, only sphingomyelin (with a T_c close to physiological temperatures) could possibility form local crystalline domains. However, the presence of equimolar levels of cholesterol 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 sub-

cellular 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.

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. The requirement for a liquid crystalline lipid matrix is more likely related to the consequent ability of lipids and proteins to diffuse rapidly in the plane of the membrane. Liquid crystalline lipids exhibit lateral diffusion rates (D_t) of 10^{-8} cm^2/s or larger, whereas membrane proteins have D_t values of 10^{-9} cm^2/s or smaller. This relates the average distance d a molecule can diffuse in a time At via the relation $d^2 = 4D_t\Delta t$. Thus, a liquid-crystalline lipid in a cell of 10 μm diameter would be able to diffuse the length of the cell within 25 s.

4.2. *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 [15,16]. The major structures (or phases) assumed are illustrated in Fig. 7, and 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 lyso-PC, 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 2 nm, is not compatible with maintenance of a permeability barrier between external and internal compartments. This immediately raises questions concerning the functional role of lipids which preferentially adopt this structure in isolation. Finally, in contrast with the situation for gel-state (crystalline) lipids, all biological membranes contain an appreciable fraction (up to 40 mol%) of lipid species which prefer the H_{II} arrangement.

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 ^{2}H-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 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. 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 Fig. 7. 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. Freeze-fracture techniques show flat, featureless fracture planes for bilayer systems,

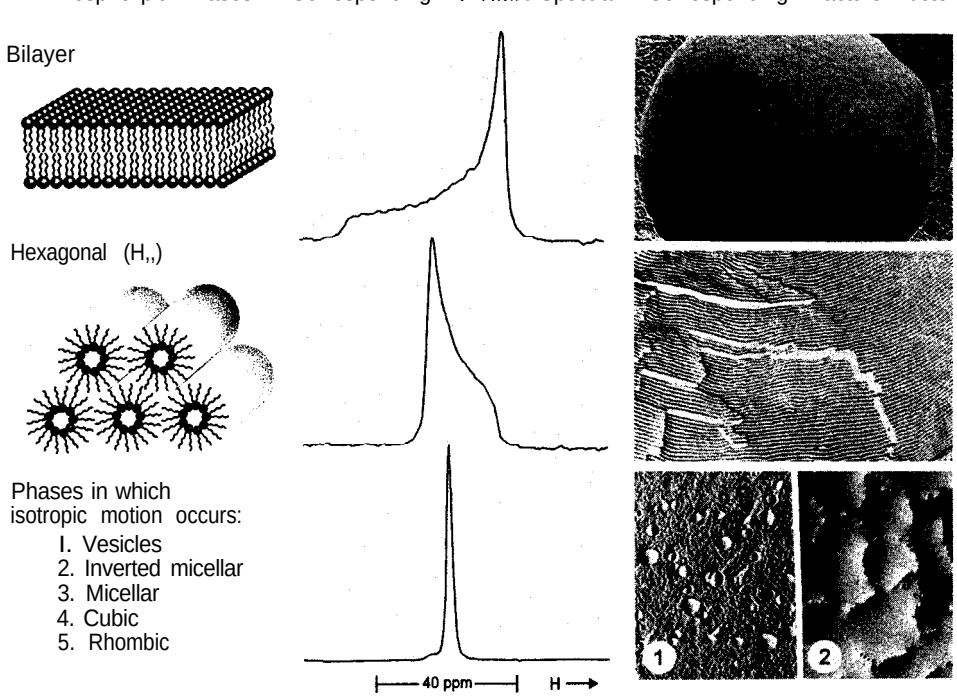


Fig. 7. ^{31}P-NMR and freeze-fracture characteristics of phospholipids in various phases. The bilayer spectrum was obtained from aqueous dispersions of egg yolk PC, and the hexagonal (H_{II}) phase spectrum from soybean PE. The ^{31}P-NMR spectrum representing isotropic motion was obtained from a mixture of 70 mol% soy PE and 30 mol% egg yolk PC. The spectra were recorded at 30°C in the presence of proton decoupling. The freeze-fracture micrographs represent typical fracture faces for the corresponding phases. 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 PC prepared by extrusion techniques and (2) large lipid structures containing lipidic particles (prepared by fusion of SUVs composed of egg PE containing 20 mol% egg PS. Fusion of the SUVs, which were prepared at pH 7, was triggered by adjusting the pH to 4).

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 [15,171 (Fig. 9). It is immediately apparent that a significant proportion of membrane lipids adopt or promote H_{II} phase structure under appropriate conditions. PE, which commonly comprises up to 35% of membrane phospholipids, is perhaps the most striking example, and particular effort has been devoted to understanding the factors which result in a predilection for the H_{II} arrangement. PE can adopt both the bilayer and H_{II} arrangements, depending on the temperature. For PEs isolated from erythrocytes, 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

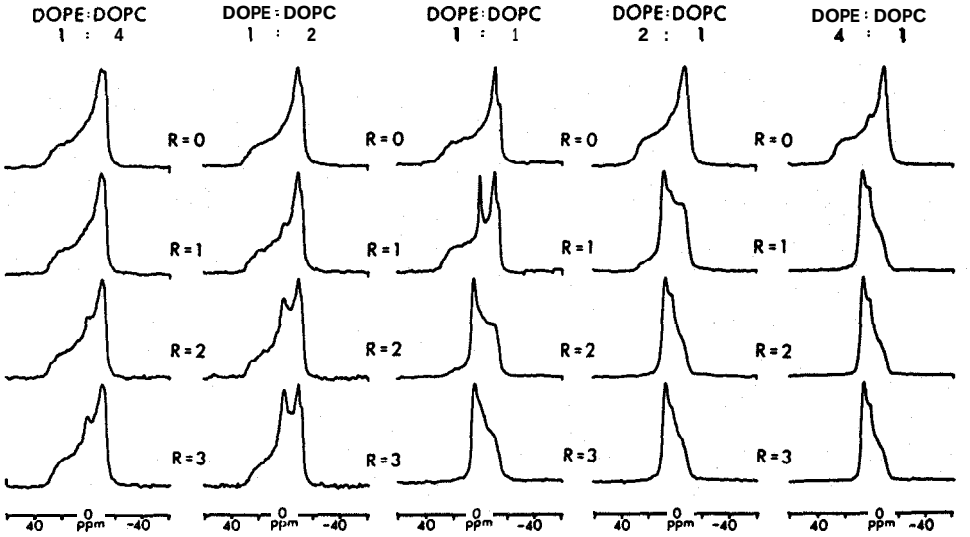


Fig. 8. Phase behavior of aqueous dispersions of dioleoyl-PC and dioleoyl-PE and the effects of cholesterol. ^{31}P-NMR spectra were acquired at 40°C for PE/PC ratios varying from 1:4 to 4:1, in the presence of varying amounts of cholesterol. The ratio R refers to the molar ratio of cholesterol to dioleoyl-PC contained in the sample. Data reproduced from Tilcock et al. [18], with permission.

T_{BH} have been observed for PE 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 characterized employing synthetic species of PE, as summarized in Table V. 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 show that mixtures of an H_{II} phase lipid (for example, PE) with a bilayer phospholipid (such as PC) result in a progressive stabilization of net bilayer structure for the whole mixture as the percentage of bilayer lipid increases, as illustrated in Fig. 8 [18]. Depending on the acyl chain composition, temperature, and head group size and charge, complete bilayer stabilization can be achieved by the addition of IO-50 mol% of the bilayer species. These systems appear to retain the ideal mixing behavior characteristic of liquid-crystalline systems. For example, in PE-PC 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. ^{2}H-NMR studies of ^{2}H-labeled varieties of these lipids indicate a homogeneous lipid composition, with no preference of the Hn-preferring PE species for the H_{II} component or of PC for the bilayer component.

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 PE-containing systems where bilayer structure has been stabilized by PC

TABLE V

The temperature (T_{BH}) of the bilayer-hexagonal H_{II} transition for some phosphatidylethanolamines

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

(Fig. 8). This effect of cholesterol is also observed in other mixed-lipid systems. The second point concerns the narrow ^{31}P-NMR peak occasionally observed in mixed-lipid systems (as in Fig. 8). Such a spectral feature cannot arise from phospholipids in H_{II} or large (diameter 2200 nm) bilayer structures, but only from phospholipids experiencing isotropic motional averaging. Lipid phases where such averaging is possible include micelles, smaller vesicles, and cubic phases. In addition, the isotropic peak could also correspond, at least in part, to a particulate feature referred to in early freeze-fracture studies as 'lipidic particles'. These structures are a general feature of mixtures of bilayer- and Hn-preferring lipids, and as noted in Section 7.1, could also represent intermediates in membrane fusion.

4.3. *Factors which modulate* lipid polymorphism

The functional roles of non-bilayer lipid structures in membranes have been investigated by characterizing the influence or divalent cations, ionic strength, pH, and membrane protein on lipid polymorphism. 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) PS and phosphatidic acid systems, and the addition of Ca^{*+} to cardiolipin triggers bilayer-Hn transitions (Fig. 9). Similar observations extend to mixed-lipid systems, where the addition of Ca^{2+} to bilayer systems containing PE and various acidic phospholipids can also trigger Hn phase formation.

PS-PE systems are perhaps the best characterized in this regard, and certain features deserve emphasis. First, in some binary phospholipid mixtures containing PS, Ca^{*+} can segregate the PS component into a crystalline (gel-phase) structure with a characteristic morphology described as 'cochleate' (as observed by freeze-fracture). In the case of PS-PE systems, the bilayer-stabilizing influence of PS is thus removed, allowing the PE to adopt the Hn organization it favors in isolation. When 30 mol% or more 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 PS, adopt the H_{II} organization. These observations have potential biological relevance, as 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 PE and PS).

4.4. The physical basis of lipid polymorphism

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 [15]. This concept is illustrated in Fig. 9, where bilayer phase lipids are proposed to exhibit cylindrical geometry, 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, hydro-

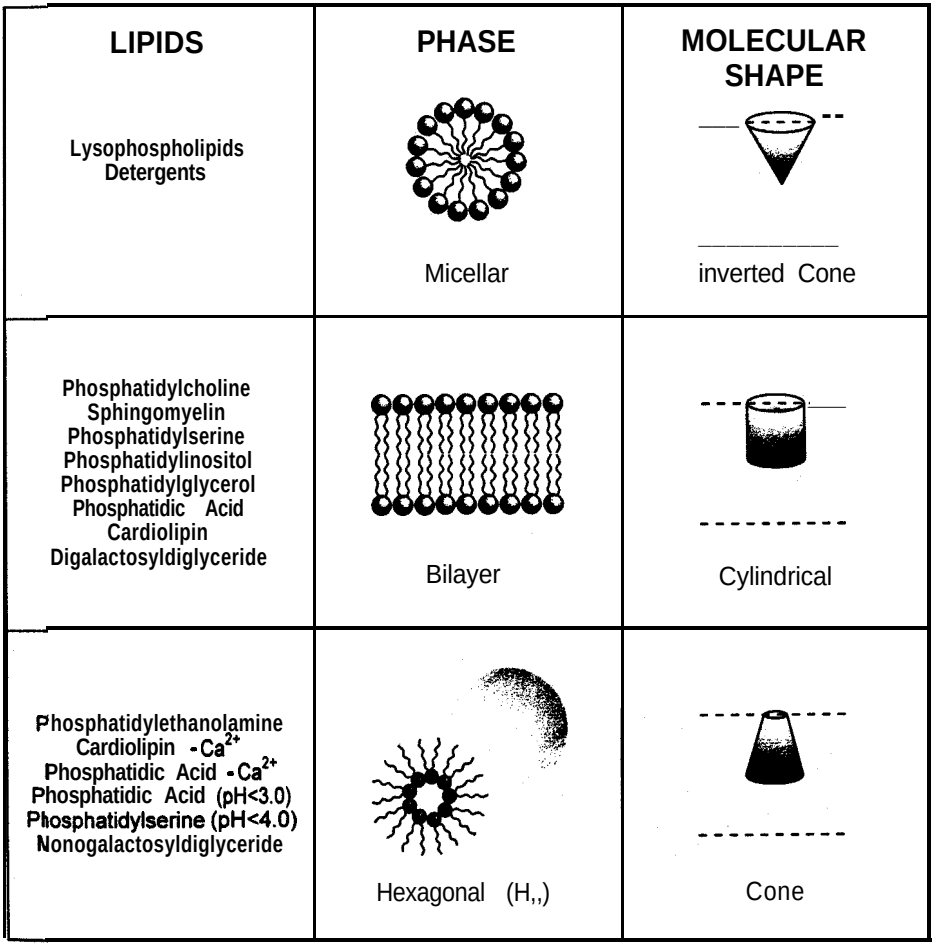


Fig. 9. Polymorphic phases and corresponding dynamic molecular shapes of lipids.

gen-bonding processes, and effects of counterions, among other possibilities. The cone shape of unsaturated PEs, for example, can be ascribed to a smaller, less-hydrated head group (in comparison with PC). Alternatively, the increased predilection of more unsaturated species of PE for the H_{II} arrangement (Table V) 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 PEs (cone shape) can adopt a bilayer structure, which may be attributed to shape complementarity [15].

More rigorous and quantitative analyses of the molecular basis of lipid polymorphism include the intrinsic radius of curvature theory developed by Gruner and colleagues (see [19] and references therein).

In summary, studies on model systems show that lipids found in biological membranes can prefer a variety of structures in addition to the bilayer phase. These structural preferences can be modulated by many biologically relevant variables, supporting the possibility that non-bilayer lipid structures play roles in membrane-mediated phenomena requiring local departures from bilayer organization. As indicated later in this chapter, membrane fusion is a most important example. It has also been proposed that the elastic stress caused by the presence of non-bilayer lipids may modulate the activity of embedded membrane proteins [19].

5. 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. 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.

5.1. 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 AC(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 AC(t) = C,(O) $\exp(-3Pt/R)$. Under conditions where the external volume is much greater than the internal trapped volume, AC(t) $= C_I(t)$ (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 calcu-

lated that the time required for release of one-half of the entrapped material ($t_{1/2}$) is 0.1 s for $P = 10^{-5}$ cm/s, whereas for $P = 10^{-10}$ cm/s, $t_{1/2}$ = 2.3 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 'unstirred' 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. Such unstirred layers can effectively reduce the solute concentration difference (AC) 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_a) 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.

5.2. *Permeability of water and non-electrolytes*

Liquid-crystalline lipid bilayers are remarkably permeable to water, which exhibits a permeability coefficient in the range of 10^{-2} to 10^{-4} cm/s [20]. 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 condition 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. Results obtained from such studies indicate that increased unsaturation of the fatty acids of the membrane increases 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.

The diffusion properties of non-electrolytes (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 glycerol across egg PC bilayers is approximately 5×10^{-6} cm/s. 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. With regard to the influence of lipid composition on the permeability of non-electrolytes, 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. 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.

5.3. Permeability of ions

Lipid bilayers are remarkably impermeable to most small ions. 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 [20]. 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, for the 100 nm diameter LW 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

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

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 μF/cm^2; thus, from the capacitance relation Q = CV (where Q is the charge and V is the transmembrane voltage), 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, and the presence of 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 PS. If we assume an area per lipid molecule of 0.6 nm^2, the resultant surface charge density σ is 8 μC/cm^2 (where C is coulombs). The resulting surface potential Φ can be calculated from the Gouy–Chapman theory [21] for a 150 mM monovalent salt buffer according to the relation $\Phi = 0.052 \sinh^{-1}(\sigma/4.5)$. This gives a negative surface potential of Φ = -69 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 in comparison with the bulk solution, resulting in a significantly lowerpH at the membrane interface and correspondingly higher H+ efflux rates.

6. *Lipid-protein interactions*

Any complete understanding of biological membrane systems necessitates a detailed understanding of the nature and influence of lipid-protein interactions that 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 or lipid polymorphism will provide the primary focus of this section. For more detailed discussion the interested reader is referred to several excellent recent reviews [22,23].

6.1. Extrinsic proteins

The interaction of extrinsic proteins with lipids has been studied using a variety of proteins, including polylysine, cytochrome c, the A, 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 penetrate partially 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 proteins on the polymorphic properties of lipids also yielded 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-PE 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 non-bilayer 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.

6.2. 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 a-helical structures. Studies on the interactions of lipids with such proteins have focused on the specificity of such lipid-protein interactions and on the physical state of the lipid. 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 possibly regulate, function. These theories were supported by early electron spin resonance 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.

A rather different picture is now generally accepted, however. First, with the exception of a possible requirement for one or two molecules of a particular lipid, lipid-protein interactions appear relatively non-specific, 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. 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 spin-label studies indicate long-lived boundary components, ^2H-NMR studies on analogous systems containing ^2H-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 exchange rapidly (exchange time $\approx 10^{-7}$ 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. This is suggested by recent ^{31}P-NMR studies which revealed different modes of protein-lipid interaction when cytochrome c was reconstituted into various anionic bilayers [24]. Furthermore, such generalizations may not hold for particular situations. The purple membrane fragments of *Hulobacterium halobium,* which contain bacteriorhodopsin, for example, exhibit a unique lipid composition distinct from the rest of the membrane.

The influence of intrinsic proteins on lipid polymorphism has been investigated by De Kruijff and co-workers [25]. Interesting features concern, first, the hydrophobic peptide antibiotic gramicidin, which spans the membrane as a dimer and which has a very strong

bilayer destabilizing capacity and even induces H_{II} phase structure in PC systems. On the other hand, glycophorin, the major asialoglycoprotein from the erythrocyte, stabilizes the bilayer structure for unsaturated PEs. These studies have been extended to signal peptides (Chapter 16). which show an ability to induce H_{II} phase structure [26] leading to the intriguing possibility that non-bilayer structures may play a role in protein insertion into, and translocation across, membranes.

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 non-specific 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 sealing proteins within the bilayer matrix and in providing an interface appropriate for membrane protein-substrate interactions.

7. *Lipids and* membrane fusion

Membrane fusion 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.

7. I. *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 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 *SW* systems will undergo fusion when incubated at temperatures in the region of their gel-liquid-crystalline transition temperature T_c. Continued recycling of sonicated dipalmitoyl-PC vesicles through $T_c = 41°C$, for example, results in fusion and formation of larger systems. Isothermal induction of crystalline structure by the addition of Ca^{2+} to PS systems results in fusion to form large crystalline cochleate structures. 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 PS, in vivo, which may act as local crystalline nucleation points for fusion. However, PS is not always present in membranes which undergo fusion, nor is Ca^{2+} able to induce crystalline cochleate-type structures for other species of (unsatu-

rated) negatively charged phospholipids. Furthermore, in more complex lipid mixtures containing PE and cholesterol, for example, there are strong indications that Ca^{2+} is not able to induce segregation of unsaturated PSs. Finally, the concentration of Ca^{2+} required to induce crystalline $PS–Ca^{2+}$ complexes is 2 mM 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. Three important observations have been made which support this hypothesis [17]. 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, which is consistent with a role of non-bilayer structure during fusion. Second, MLV systems composed of lipid mixtures such as PEs and charged lipids such as PSs or phosphatidic acid form H_{II} structures on the addition of Ca^{2+}. *SW* or LW systems with these lipid compositions first fuse to form larger lamellar systems exhibiting lipidic particle structures, 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. These observations have led to a general hypothesis that factors which tend to induce non-bilayer (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} 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 non-bilayer 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 non-bilayer intermediate, as shown in Fig. 10. More quantitative support for the proposal that intermediates in bilayer-non-bilayer phase transitions also provide intermediate structures for membrane fusion comes from the elegant thermodynamic analyses and cryo-electron microscopy studies performed by Siegel and co-workers [27]. Specifically, these authors provide convincing evidence that bilayer-non-bilayer transitions proceed through either inverted micellar intermediates or stalk structures (the latter being energetically more feasible), both of which eventually form transient 'interlamellar attachment sites', which are lipid cylinders formed between adjacent bilayers. These structures, illustrated in Fig. 10, are thought to correspond to the lipidic particle structure observed by freeze-fracture electron microscopy (see Fig. 7). The interlamellar attachment sites, which have been visualized by cryo-transmission electron microscopy during fusion processes, are of sufficient curvature to give rise to the isotropic resonances observed by ^{31}P-NMR (Section 4.2).

7.2. *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

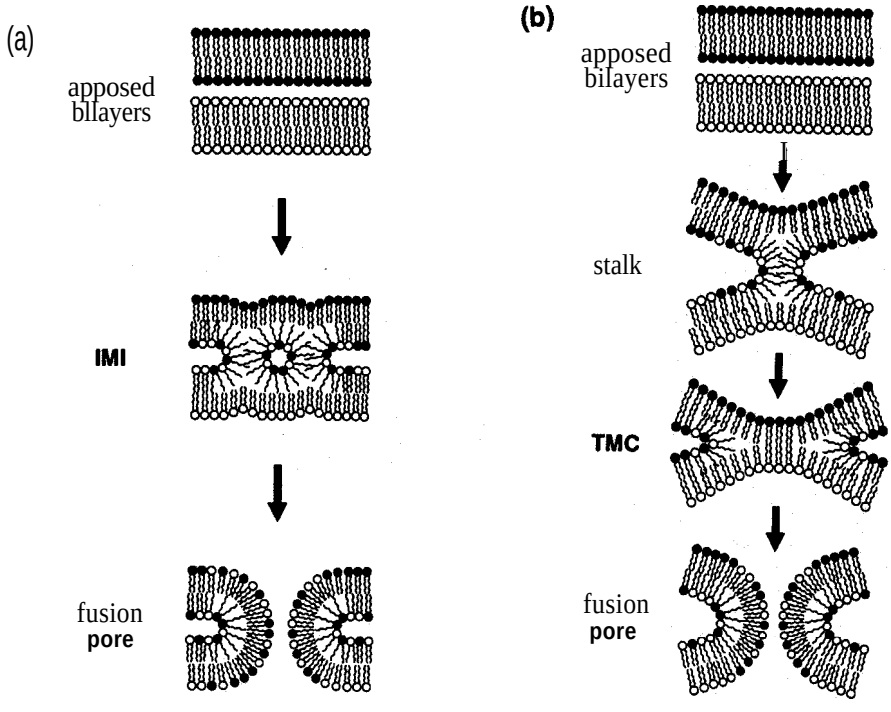


Fig. 10. Two possible mechanisms of membrane fusion, involving inverted micellar intermediates (a), and stalk intermediates (b). The interlamellar attachment (ILA) and fusion pore are equivalent. See Ref. [27] for details. (Modified from Siegel 1271, with permission).

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. 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 of the chromaffin cell is likely composed primarily of PS and PE, whereas the outer (also cytosolic) monolayer of the secretory granule membrane is enriched in PC and sphingomyelin. 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 non-bilayer structures. In another system, myoblast cells (which fuse to form the multinucleated muscle fibers) have been studied. Such fusion, which is also Ca^{2+}-dependent, may rely on the transmembrane distribution of PE and PS, 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. Freeze-fracture work suggests that the striated patterns characteristic of tight junc-

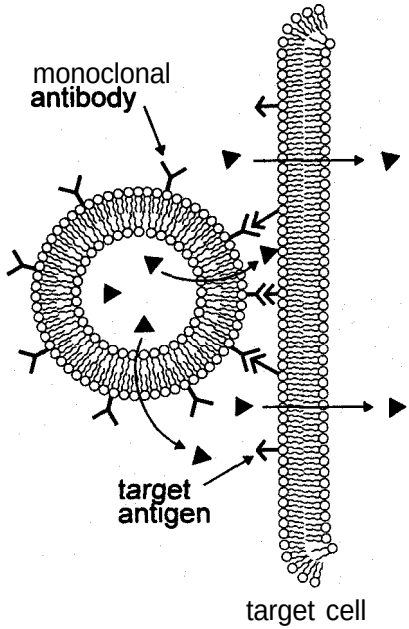


Fig. 11. The potential delivery of biologically active materials (solid triangles) encapsulated in membrane vesicles. Tissue-specific antibodies (Y) are covalently attached to the surface of the vesicle and enable the targetting of entrapped material.

tion assemblies may correspond to long, inverted lipid cylinders similar to those comprising the H_{II} phase structure [28]. Similar states of arrested fusion may correspond to the contact sites between the inner and outer membranes of mitochondria and *E. coli.*

8. 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 [29]. Natural membrane lipid components such as PC are remarkably non-toxic and non-immunogenic 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 Fig. 11.

The many difftculties 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 exist, including the reversed-

phase evaporation protocol and the extrusion protocol outlined previously, which allow maximum trapping efficiencies in the range of 30-50% of available drug. In addition, new procedures have become available that rely on transmembrane pH gradients across LUV membranes and that allow the rapid, efficient encapsulation of lipophilic, cationic drugs such as the anticancer drugs doxorubicin and vincristine. These procedures allow encapsulation efficiencies approaching 100% and extremely high interior drug concentrations of 300 mM or higher. 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 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 can be circumvented by inclusion in the liposome of gangliosides such as G_{M1}, or of hydrophilic polymers (such as polyethyleneglycol) covalently attached to the headgroup of certain lipids, which minimize interactions with plasma proteins. However, other significant problems 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 clinical importance. These advances are largely based on the finding that liposomal encapsulation of anticancer agents (such as doxorubicin or vincristine) or antifungal agents (such as amphotericin B) can reduce the toxicity associated with the drug while maintaining or even increasing efficacy [29,30]. Other applications take advantage of the natural targeting of liposomes to fixed and free macrophages of the reticuloendothelial system. An example is given for treatment of parasites which reside in the macrophages and which are difficult to eliminate 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 *Leishmania.* An advantage of this method of treatment is that the dose levels needed are much lower than otherwise required. As summarized elsewhere [29], extensive clinical trials are being conducted on these and other liposomal formulations.

A novel extension of conventional liposome drug delivery is the use of lipid/DNA mixtures to deliver plasmid constructs for gene therapy applications. The observation that vesicles composed of equimolar dioleoyl-PE and a cationic lipid can be employed to deliver viable gene constructs into cells in vitro was first reported in 1987 [31]. The precise mechanism is still unclear, however, it is known that the cationic lipid component binds to DNA causing the nucleic acid to condense into a particle and that dioleoyl-PE is normally required to maximize transfection activity [32]. This has been demonstrated in studies where a plasmid containing the gene for bacterial β-galactosidase was introduced into cultured cells using a cationic lipid mixed with dioleoyl-PE or dioleoyl-PC. Gene expression was followed by measuring β-galactosidase activity. The dependence on charge ratio partly reflects the need to have sufficient cationic lipid present to condense DNA. The role of dioleoyl-PE is widely believed to be associated with this lipid's ability to destabilize bilayer structure and subsequent involvement in membrane fusion events

(Section 7). Lipid/DNA complexes are taken into cells by endocytosis. When dioleoyl-PE is present the endosome is disrupted, releasing some DNA into the cytoplasm before lysosomal degradation occurs. A proportion of the cytoplasmic DNA diffuses into the nucleus where transcription can take place. Replacing dioleoyl-PE with the non-fusogenic lipid dioleoyl-PC reduces the efficiency of transfection, even though DNA is condensed into a particle and endocytosed by cells to the same extent for both delivery systems. Lipid/DNA complexes are being developed for use in vivo [33] and offer several advantages over the more common viral vectors including their low immunogenicity.

9. Future directions

The physical properties of membrane lipids are increasingly well understood. The relation between these physical properties and the functional roles of lipids remain relatively obscure, however. General roles of lipids in maintaining the membrane bilayer permeability barrier are clear. Similarly, the ability of certain classes of lipids to adopt non-bilayer structures are very probably of utility in inter-membrane interactions such as fusion, which require local departures from the bilayer organization. These abilities could be satisfied by a relatively limited subset of the lipids actually present in membranes. Important additional functions which require clarification include the detailed roles of lipids in establishing and modulating membrane permeability and membrane protein function as well as their roles in transbilayer signalling phenomena.

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