# THE DYNAMICS OF MEMBRANE STRUCTURE

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## I. INTRODUCTION

One of the most notable features of all living cells is that the relationship between their domestic activities and the external environment is regulated by a membranous barrier. Moreover, many of the internal cellular functions are performed within compartments bounded by membranes. Membranes forming the cell boundary as well as the internal membranes are all lipoprotein structures. The arrangement of the lipid and protein in the membranes was conceived originally as a static array of metabolically inert molecules. Early models of membrane structure constructed on this principle appeared at first to provide a reasonable account of the properties of some cell membranes, and the interpretation of membrane architecture from electron microscopic studies seemed to support these models. Contemporary views of membrane structure, however, are rather different. They have been shaped largely from a better understanding of the properties of the constituent molecules and of their mutual interactions which create the particular microenvironment that exists within the membrane structure. Some cell membranes are depicted as consisting of a dynamic array of molecules highly mobile within the structure and constantly interacting with molecules within the membrane as well as those of the surrounding media. Many processes conducted in and around cell membranes appear to depend on specific interactions between the different components of the membrane.

In this review, we first examine the chemical composition and physical properties of the molecules found in biological membranes. We next discuss model membrane systems including reconstituted systems and then various physical and chemical studies of natural membranes. This is linked with the biochemical regulation processes involved in membrane structure. Finally, we attempt to integrate these results to provide a picture of the dynamics of biomembrane structures and possible mechanisms whereby this may be regulated.

## II. THE CONSTITUENTS OF CELL MEMBRANES

Biological membranes consist of proteins, lipids, carbohydrates cholesterol, ions,

TABLE I

**Chemical Composition of Cell Membranes Arranged in Order of Increasing Protein:Lipid Ratio**

| Membrane | Protein (%) | Lipid (%) | Carbohydrate (%) | Weight fraction of protein | Ratio of protein to lipid |
|---|---|---|---|---|---|
| Plasma membranes | | | | | |
| Myelin | 18 | 79 | 3 | 0.18 | 0.23 |
| Blood platelets | 33—42 | 51—58 | 7.5 | 0.4 | 0.7 |
| Mouse liver cells | 46 | 54 | 2—4 | 0.46 | 0.85 |
| Human erythrocytes | 49 | 43 | 8 | 0.49 | 1.1 |
| Amoeba | 54 | 42 | 4 | 0.54 | 1.3 |
| Rat liver cells | 58 | 42 | (5—10)* | 0.58 | 1.4 |
| HeLa cells | 60 | 40 | 2.4 | 0.6 | 1.5 |
| Nuclear envelope of rat liver cells | 59 | 35 | 2.9 | 0.59 | 1.6 |
| Retinal rods, bovine | 51 | 49 | 4 | 0.51 | 1.0 |
| Mitochondrial outer membrane | 52 | 48 | (2—4)* | 0.52 | 1.1 |
| Sarcoplasmic reticulum | 67 | 33 | — | 0.67 | 2.0 |
| Chloroplast lamellae, spinach | 70 | 30 | (6)* | 0.7 | 2.3 |
| Mitochondrial inner membrane | 76 | 24 | (1—2)* | 0.76 | 32 |
| Gram-positive bacteria | 75 | 25 | (10)* | 0.75 | 3.0 |
| *Halobacterium* purple membrane | 75 | 25 | | 0.75 | 3.0 |

* Deduced from analyses.

After Guidotti, G., *Annu. Rev. Biochem.*, 41, 731, 1972.

and water, in variable proportions (see Table 1). As the sugar residues are invariably attached to the protein or the lipid components, we shall consider the membrane molecules to consist of proteins (and glycoproteins) and of lipids (and glycolipids) with some membranes containing appreciable amounts of cholesterol or other sterol-type molecule.

## A. The Lipids

The hydrophobic character of cell membranes is due almost entirely to the lipid component, and most lipids, in monomeric form, are insoluble in water. Virtually complete extraction of membrane lipids can be achieved with suitable nonpolar organic solvents. Solvent treatment causes denaturation of most membrane proteins which can be conveniently separated from the solvated lipids. Many techniques have been devised to analyze and characterize the lipid component of biological membranes, and this has been the principal reason why, in contrast to membrane proteins, we have a more complete understanding of the structure of theses components.

### *1. Lipid Structure*

The lipid components of cell membranes consist of phospholipids (phosphoglycerides and sphingomyelin) glycolipids and neutral lipids. Associated with the lipid molecules are fatty acid residues of varying chain length and number of unsaturated bonds.

The phosphoglycerides, a major class of membrane lipids, are based on the glycerol molecule, in which two hydroxyl groups are esterified with long-chain fatty acids. A hydrated group is esterified to the third carbon atom of the glycerol phosphodiester linkage. Usually phosphoglycerides from biological membranes consist of one saturated and one unsaturated fatty acid, the latter generally situated on the central carbon atom of the glycerol, between the saturated acid at one end and the polar group at the

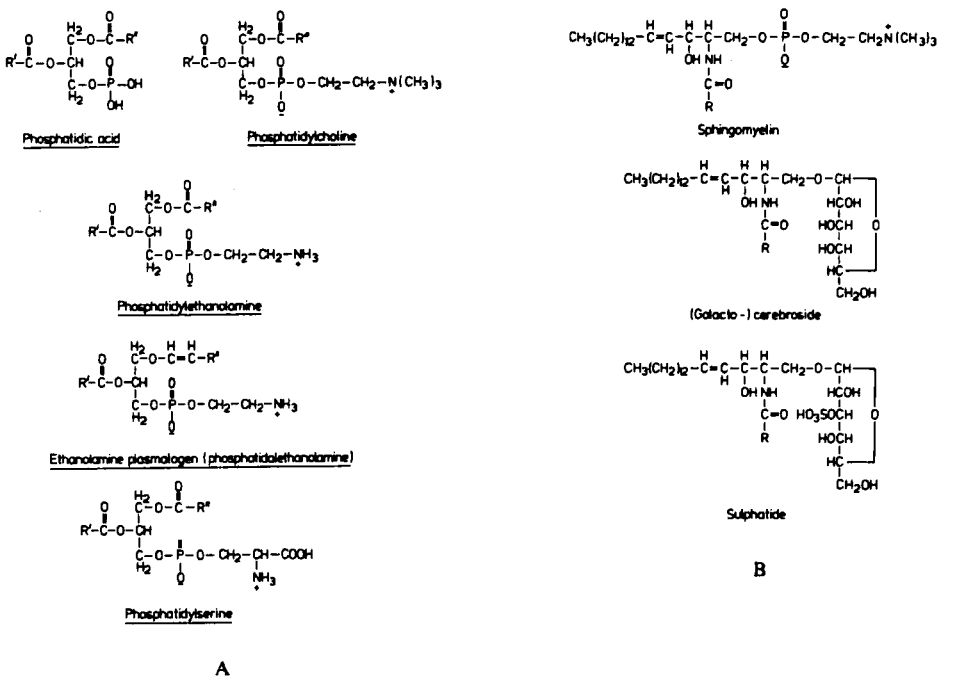



FIGURE 1. Structure of some of the major phospholipid classes (A) and sphingolipids (B).

other.[2] A notable exception to this arrangement is the phospholipid of the alveolar membrane of lung tissue which consists predominantly of dipalmitoyl lecithin.

The type of polar group associated with the phospholipids defines the phospholipid class. All of them, as the name implies, contain a phosphate group, but additional groups shown in Figure 1A are also present. The lecithins are characterized by the presence of a choline group attached to the phosphate. Other groups include amines of similar structure to choline (as in phosphatidylethanolamine); the amino acids serine and threonine (in phosphatidylserine and phosphatidylthreonine); various modifications of the glycerol molecule, sometimes with one or even two additional phosphate groups attached to it: as in 1-phosphatidylinositol, 1-phosphatidyl(inositol-4-phosphate), and 1-phosphatidyl (inositol-4,5-diphosphate).

The second major class of membrane lipids is the sphingolipids; these are based on the molecule of a long-chain amino alcohol — sphingosine (Figure 1B). Although the basic design of the phospholipids are similar, they differ from phosphoglycerides in a number of respects. The first long-chain component is invariably a 15:1 hydrocarbon which is linked to the base by a carbon-carbon bond rather than an ester bond

typical of the phosphoglycerides. In addition, a hydroxyl group is retained. To the second carbon of the sphingosine is bonded one of several fatty acids by an amide

rather than an ester bond. The polar group may be a phosphate-plus-choline-structure

(sphingomyelin), a simply hydroxyl (the ceramides), or a complex polysaccharide (the gangliosides).

Thus glycolipids (sugar-containing lipids) differ from sphingomyelin in the nature of the unit that is linked to the primary hydroxyl group of sphingosine. The simplest glycolipid is cerebroside, containing only one sugar residue, either glucose or galactose. More complex glycolipids, such as gangliosides, contain a branched chain of as many as seven neutral and amino sugar residues.

Neutral lipids are present in varying amounts in cell membranes, and they are usually more abundant in the plasma membrane. Unesterified cholesterol is the predominant neutral lipid of membranes of eukaryotic cells (but not in prokaryotic cells), and neutral glycerides, cholesterol esters, and free fatty acids are relatively minor membrane lipid components.

### *2. Lipids of Cell Membranes*

There have been numerous attempts to define the variations in lipid composition observed in different membranes (see References 3 to 7 for reviews). Rouser and coworkers[3] have summarized the analyses of many membrane systems as follows:

1. All animal cell membranes contain phospholipids, and the phospholipid classes represented in each particular membrane are usually characteristic. Table 2 shows the major phospholipid classes present in rat liver cell membranes. The same classes of phospholipids are found in vertebrates and invertebrates. Some membranes contain glycolipids whereas others do not. Only certain membranes contain sterol.
2. Plasma (cell surface) membranes, membranes of the endoplasmic reticulum, nuclear membranes, and mitochondrial membranes from the same species have different compositions. All differ quantitatively and to some extent qualitatively in the classes of lipids present. For example, plasma membranes or elaborations of these (such as myelin) appear to contain most of the glycolipids of the cell. Even where qualitative differences in distribution of lipid classes are not seen, as in endoplasmic reticulum and nuclei, the quantitative distribution of lipid classes is different and characteristic for each membrane.
3. Plasma membranes, as shown by studies of mammalian erythrocytes, exhibit large species variation in composition. The proportions of the different phospholipids vary greatly between species, and the total amount as well as the types of both ceramide polyhexosides and gangliosides is very different in different species. Data from whole organs indicate that plasma membrane from different cell types of the same species may vary in composition.
4. Mitochondria from bovine heart, kidney, and liver contain diphosphatidylglycerol (cardiolipin), phosphatidylcholine, and phosphatidylethanolamine as the major phospholipids in the approximate molar ratio 1:4:4, but the inner and outer membrane of mitochondria differ in composition. The outer membrane contains virtually all the cholesterol of the mitochondrion and appears to be devoid of cardiolipin. On the other hand, mitochondria from different organs of one species appear to have the same phospholipid distribution, and species variation in mitochondrial phospholipid distribution among vertebrates is, at most, slight.
5. The fatty acid composition of each class of lipids from different organelles and organs of one species, as well as from different species, is variable (Table 3). This is true even when the classes of lipids are the same in the different structures. Individuality is thus expressed most clearly in differences in fatty acid composition.

TABLE 2

**Phospholipid Composition of Liver Cell Membranes**

| | Plasma membrane | Nuclear membranes | Endoplasmic reticulum | | Golgi membranes | Mitochondrial membranes | | Lysosomal membranes |
|---|---|---|---|---|---|---|---|---|
| | | | Rough | Smooth | | inner | outer | |
| Phosphatidyl choline | 34.9 | 61.4 | 60.9 | 55.4 | 45.3 | 45.4 | 49.7 | 33.5 |
| Phosphatidyl ethanolamine | 18.5 | 22.7 | 18.6 | 21.5 | 17.0 | 25.3 | 23.2 | 17.9 |
| Phosphatidyl inositol | 7.3 | 8.6 | 8.9 | 6.7 | 8.7 | 5.9 | 12.6 | 8.9 |
| Phosphatidyl serine | 9.0 | 3.6 | 3.3 | 1.9 | 4.2 | 0.9 | 2.2 | 8.9 |
| Phosphatidyl glycerol | 4.8 | — | — | — | — | 2.1 | 2.5 | — |
| Phosphatidic acid | 4.4 | 1.0 | 1.0 | 1.9 | — | 0.7 | 1.3 | 6.8 |
| Cardiolipin | Trace | 0 | — | — | — | 17.4 | 3.4 | 6.8 |
| Lysophosphatidylcholine | 3.3 | 1.5 | 4.7 | — | 5.9 | — | — | 0 |
| Lysophosphatidylethanolamine | — | 0 | 0 | — | 6.3 | — | — | — |
| Sphingomyelin | 17.7 | 3.2 | 3.7 | 12.3 | 12.3 | 2.5 | 5.0 | 32.9 |

*Note:* All values expressed as moles percent of total phospholipid.

From White, D. A., in *Form and Function of Phospholipids*, Ansell, G. B., Hawthorne, J. N., and Dawson, R. M. C., Eds., Elsevier, Amsterdam, 1973, 441.

TABLE 3

**Fatty Acid Composition of Lipids From Cell Membranes[a]**

| Membrane | Percent of total fatty acids | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:2 | 20:3 | 20:4 | 22:6 |
| Human erythrocytes | | | | | | | | | | |
| Whole membrane | 1.1 | 29.5 | 2.4 | 17.1 | 13.5 | 3.9 | 1.4 | — | 1.9 | |
| PC | — | 34.7 | — | 13.8 | 21.1 | 21.9 | — | 1.0 | 6.7 | |
| PE | 0.2 | 18.9 | 0.6 | 8.0 | 25.2 | 7.0 | 0.1 | 1.0 | 21.9 | |
| SM | 1.6 | 16.0 | 2.3 | 9.7 | 39.6 | 11.5 | — | 1.9 | 1.4 | |
| Rat liver | | | | | | | | | | |
| Cell membrane | | | | | | | | | | |
| PC | 10.5 | 30.2 | 0.8 | 29.8 | 9.6 | 15.3 | | | 11.6 | |
| PE | 0.2 | 26.0 | 0.3 | 31.9 | 6.5 | 12.4 | | 15.4 | | |
| SM | 0.2 | 18.7 | 0.1 | 39.2 | 3.7 | 4.2 | | | 12.8 | |
| Mitochondria | | | | | | | | | | |
| PC | 0.4 | 27.0 | 3.9 | 21.6 | 13.0 | 12.4 | — | 1.3 | 17.7 | 2.9 |
| PE | 0.3 | 26.6 | 263.2 | 27.3 | 12.0 | 5.4 | — | — | 22.0 | 3.2 |
| CL | 0.2 | 7.0 | 7.6 | 3.6 | 19.9 | 58.8 | — | 1.2 | 1.8 | |
| Mitochondrial outer membrane | | | | | | | | | | |
| PC | 0.5 | 31.5 | 4.6 | 26.1 | 13.3 | 11.0 | — | 1.0 | 10.7 | 1.5 |
| PE | 0.2 | 30.6 | 2.2 | 26.1 | 8.2 | 6.3 | — | 0.3 | 21.4 | 4.8 |
| Inner membrane | | | | | | | | | | |
| PC | 0.5 | 27.8 | 3.9 | 20.5 | 13.6 | 14.0 | — | 1.4 | 15.8 | 2.5 |
| PE | 0.5 | 24.8 | 1.8 | 27.7 | 8.8 | 4.7 | — | 0.4 | 23.8 | 7.5 |
| CL | 0.5 | 7.7 | 6.2 | 4.8 | 19.2 | 57.8 | — | 2.4 | 14.0 | — |
| Microsomes | | | | | | | | | | |
| PC | 0.8 | 24.5 | 3.3 | 21.0 | 12.3 | 17.7 | — | 1.2 | 15.8 | 2.9 |
| PE | trace | 22.6 | 2.3 | 23.4 | 9.8 | 10.3 | — | trace | 23.1 | 7.2 |
| Smooth endoplasmic reticulum | | | | | | | | | | |
| PC | 0.4 | 28.6 | 3.1 | 26.5 | 10.6 | 14.9 | — | 1.4 | 14.0 | 0.7 |
| PE | 0.2 | 26.1 | 2.7 | 25.5 | 9.9 | 10.9 | — | 0.7 | 15.8 | 7.2 |
| Rough endoplasmic reticulum | | | | | | | | | | |
| PC | — | 22.7 | 3.6 | 22.0 | 11.1 | 16.1 | — | 1.8 | 19.7 | 2.9 |
| PE | 0.3 | 21.5 | 1.5 | 23.5 | 8.3 | 10.2 | — | — | 22.9 | 11.3 |

*Note:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; SM, sphingomyelin.

[a] Data from References 3, 9, and 10.

One of the most remarkable and, as yet, unexplained features of membrane phospholipid composition is the fact that the major lipid classes represented in each particular membrane is characteristic of that membrane. This prevails despite the varying and high metabolic turnover of membrane phospholipids and the rapid exchange mediated by the phospholipid exchange proteins (see Section V.B.2).

## B. The Proteins

The lipids were for a long time the principal focus of the biochemical examination of membranes because they could be analyzed by well-established techniques. The study of the membrane proteins has, however, posed a number of major problems. Membrane proteins are, in the main, insoluble in water under normal circumstances. Upon extraction of the lipids, the proteins can form recalcitrant aggregates and lose their biological activity. This is because the interaction of membrane proteins with polar solvents is not energetically favorable compared with their interaction with other membrane components which presumably provide the necessary hydrophobic environment required to preserve their native conformation. It is therefore difficult to study membrane proteins by techniques developed for the examination of water-soluble proteins although some extrinsic membrane proteins may be regarded as exceptional (cytochrome c, for example). Membrane proteins are, however, now currently under intensive investigation. The characterization of some membrane proteins has progressed considerably, and the amino acid sequence of many proteins have now been studied in detail (for reviews, see References 1 and 11).

The study of membrane proteins involves (1) the release of proteins by "solubilization" of membranes, (2) the isolation of the purified membrane proteins and, (3) the characterization of these proteins. Many purified proteins have been used for studies of protein-lipid interactions in model systems or in reconstituted systems, and we shall examine some of these later.

A number of proteins can often be removed from biological membranes by relatively mild extraction procedures, suggesting that they may not be integrated firmly into the structure but merely adsorbed to the surface.[1] This poses the question as to which proteins are truly membrane-bound, in the sense that they constitute part of the structure or are normal functional components of the membrane, and which proteins are adventitiously adsorbed or are simply contaminants of the preparation. Ideally, one would include all proteins that associate in a *specific* manner with the membrane in vivo and exclude those that are nonspecifically adsorbed. In practice, specific associations could be defined as those that cannot be ruptured by repeated washing of the membrane with physiological solutions. A case in point is that of the red cell membrane which adsorbs small amounts of hemoglobin, although it is likely that membranes from other sources are similarly contaminated with less conspicuous proteins. This problem is particularly serious when it is recognized that proteins normally free in the cytoplasm may adsorb nonspecifically to membranes on manipulation of the ionic conditions and, in the absence of evidence to the contrary, are likely to be regarded as specific membrane proteins. An example is the enzyme glyceraldehyde 3-phosphate dehydrogenase which adsorbs to erythrocyte membranes when lysed with buffers of low ionic strength but not when membranes are prepared by lysis under isotonic conditions.[12] A further difficulty is experienced when soluble proteins become trapped inside membrane vesicles that form during tissue homogenization; no amount of washing is capable of removing these proteins.

### *1. Extraction of Membrane Proteins*

The release of proteins from membranes is obtained by "solubilization" methods.[11] A suspension of membranes can be made to clarify and form precipitates when the

sample is centrifuged under conditions known to sediment the membranes. This indicates a sharp reduction in the particle size. Whether the proteins and lipids have been reduced to their fundamental molecular state at this stage cannot be inferred. Thus terms like "disaggregation" or "dispersal" may be more appropriate than "dissolution".

There are various agents and procedures for solubilization of membrane proteins:[13] alteration in ionic conditions (low ionic strength or high ionic strength, combined sometimes with a change of pH or in combination with metal chelating compounds), aqueous denaturants (guanidine-HCl, urea), organic solvents (e.g., 2-chloroethanol, pyridine, acidified phenol, organic acids, butanol, and pentanol), detergents, digestion of membranes with proteases, sonication, or succinylation. Some selected examples are given in Table 4. Various products are obtained after solubilization of membranes. For example, membranes may be finely dispersed in small vesicles or lipoprotein dispersions (e.g., by ultra sonication or using detergents).

The conditions that are required to extract particular proteins from membranes can provide useful information about the nature of the interaction with other membrane components. Treatment of certain membranes with salt (0.1 to 0.5 M NaCl) is sufficient to release some of the protein components[15] suggesting that electrostatic binding is the predominant force involved in the interaction. Selective extraction procedures have been applied to the human erythrocyte membrane to resolve the major protein components of this membrane.[16] This membrane contains six major protein components comprising about two thirds of the total membrane protein. The remaining protein is distributed amongst nine other bands that can be resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Spectrin, a high molecular weight protein located on the cytoplasmic surface of the membrane, together with another, as yet, uncharacterized protein, is released from the membrane when washed with alkaline, low-salt buffer containing a metal chelating compound. This treatment does not alter the density of membrane-associated particles observed in freeze-fracture replicas of the membrane suggesting that these two proteins do not penetrate extensively into the hydrocarbon region of the membrane but are simply adsorbed by coulombic forces to the surface. Subsequent exposure of the membrane to M NaCl releases almost quantitatively the enzyme D-glyceraldehyde 3-phosphate dehydrogenase, and this treatment is associated with a vesiculation of the membrane in which the remaining components are likely to have undergone considerable rearrangement. After this treatment, about half of the total protein remains but no membrane-associated particles are visible on the fracture faces. Extraction of these vesicles with 33% aqueous pyridine releases sialoglycoprotein, and the remaining two major proteins require detergents to remove them from the bulk of the membrane lipids. One of the residual proteins that remain after the aqueous pyridine extraction is the so-called band 3 glycoprotein because of its position on the gel electrophoretogram.[15] Band 3 glycoprotein is believed to be involved in several different functions including anion permeability,[17,18] sugar,[19,20] and water[21] transport and to act as a receptor for concanavalin A[22]. The protein has been extracted and purified from human erythrocytes using a combination lithium diiodosalicylate treatment and detergent solubilization, lectin affinity chromatography, and gel filtration.[23]

A general classification of membrane proteins has been devised on the basis of the method required to extract them from membrane structures. Proteins that can be dislodged from the membrane by relatively mild procedures such as the addition of salts or chelating agents are referred to as extrinsic or peripheral proteins. It is inferred that such proteins are bound to the surface of membrane predominantly by electrostatic and perhaps hydrogen bond interactions. Other proteins, the so-called integral proteins, are bound much more tenaciously and can be separated only by detergents or

TABLE 4

A Selection of Techniques and Agents Used for Solubilization of Membrane Proteins

| Agent or technique | Protein and source | Comments |
|---|---|---|
| Osmotic shock | High affinity binding proteins, Gram-negative bacteria | Proteins released are probably perycytoplasmic or loosely bound to membrane rather than integral components of membrane |
| Distilled water | Spectrin from ghosts of human red blood cells | Membrane completely solubilized only if ghosts have been thoroughly deionized |
| Low ionic strength | ATPase, *Streptococcus faecalis* | Enzyme solubilized by repeated washes with 1 mM Tris-HCl buffer in absence of divalent cations |
| High ionic strength | Proteins from ghosts of human red cells | 0.8 M NaCl solubilizes about 40% of membrane protein |
| Chelating agents | Spectrin from ghosts of human red blood cells | 5 mM EDTA, low ionic strength buffer, plus β-mercaptoethanol |
| | A major protein of rat liver plasma membrane | 1 mM EDTA |
| | A major protein from chloroplast membrane resembling the $F_1$ ATPase of mitochondria | 1 mM EDTA |
| Triton X-100, | Acetylcholinesterase electroplax electric organ | 0.5% w/w detergent solution for 4 gh, at 4°C; sonication before detergent treatment; enzyme partially inactivated |
| Cetyltrimethyl-ammonium bromide (CTAB) | Rhodopsin | 40 mM CTAB |
| Deoxycholate | Acetylcholine receptor, electropax electric organ | 1% w/w detergent solution; acetyl cholinesterase also solubilized |

Adapted from Chavin, S. I., *FEBS Lett.*, 14, 269, 1971.

organic solvents, in which case the protein may be contaminated by some of the lipid components of the membrane and can be regarded for most purposes as a lipoprotein complex.

### 2. *Fractionation of Membrane Proteins*

The fractionation of membrane proteins is generally accomplished using suitable adaptations of techniques developed for soluble proteins. Direct application of conventional techniques is not always practicable, however, because of the water-insoluble nature of membrane constituents.[11] The procedure whereby extracted proteins can be resolved on the basis of molecular weight using sodium dodecylsulfate polyacrylamide gel electrophoresis has already been mentioned. Other methods include the fractional precipitation with ammonium sulphate or other compounds used to "salt out" proteins which can be effective even with detergent-solubilized proteins. Centrifugation (differential, rate zonal, or equilibrium density centrifugation) techniques are widely used for fractionation because the dispersed membrane components may be heterogenous in size and density. Proteins dispersed with solvent, detergent, or denaturant may be dialyzed against water to remove these agents, but the proteins usually precipitate under these conditions. Sometimes membrane proteins may be dispersed free of lipids

by nonpolar solvents like 2-chloroethanol or detergents such as sodium dodecylsulfate and separated from the lipids by gel filtration, gel electrophoresis, or ion-exchange chromatography. Details of the application of such techniques are given in References 24 and 25.

The fractionation procedures may give information about characteristics of membrane proteins, such as the molecular size (gel filtration chromatography), the density and size (centrifugation), and the charge and/or molecular weight (polyacrylamide gel electrophoresis). Often a combination of these methods must be employed because of the unique features encountered in some membrane proteins. The glycoproteins, for example, often exhibit anomalous mobility characteristics in sodium dodecylsulfate polyacrylamide gel electrophoresis due primarily to the bulky carbohydrate residues. Other proteins include those with unusual charge or conformation and peptides containing unreduced sulphydryl groups. This behavior can sometimes be avoided by prior treatment with sulphydryl reducing agents and in other cases by comparing the mobility in gels that are cross-linked to varying degrees with the free electrophoretic mobility of the detergent complex.

An important question with regard to the protein composition of membranes is the number of different polypeptide species associated with particular membranes. Operationally, the extraction of all proteins from membranes and their dissociation into subunits by sodium dodecylsulfate followed by electrophoresis in polyacrylamide gels in the presence of detergent has proved a versatile and reliable method. Examination of the spectrum of polypeptides obtained from a variety of different subcellular membranes has indicated that certain peptides are found only in particular cell membranes and do not appear in other morphologically distinct membranes during the life of the cell; the relative amount of such peptides may vary considerably with time or stage of differentiation of the membrane and in some circumstances may not be detected at all. Such changes are believed to reflect different functional activities of membranes or represent stages in the differentiation of the structure.

There is a considerable heterogeneity in the molecular size and electrophoretic mobility of membrane proteins. The molecular weights of membrane polypeptides vary widely, ranging from below 20,000 to more than 200,000 daltons per chain. Indeed, some of the largest polypeptides encountered in biological systems are components of the cell membranes. The number of polypeptides in different membranes can also vary quite widely. Certain viruses, the arboviruses for example, have only one discernible polypeptide, possibly a glycoprotein, in their lipoprotein envelope.[26] An unusual bacterial strain, *Bacillus* PP, obtained from a culture of *B. megaterium* KM, has a single polypeptide component of molecular weight 32,000 daltons.[27] It constitutes at least 90% by weight of the ghost membrane protein and has an electrophoretic mobility indistinguishable from a minor component of the presumed parent strain. It may represent an overproduction of the specific gene product (usually the electrophoretic patterns of polypeptides from bacterial species show a multitude of membrane components). Other membranes that appear to have only one prominent polypeptide component include the rod outer segment membranes of the bovine retina in which rhodopsin predominates,[28] and sarcoplasmic reticulum in which $Ca^{2+}$-activated ATPase is the major protein component.[29]

The spectrum of polypeptides from other cell membranes may be considerably more complex than that observed in retinal rod outer segment disc and sarcoplasmic reticulum membranes. Membranes of rat liver mitochondria, for example, exhibit 23 polypeptides in the inner and 12 in the outer membrane, all of which are different, with the possible exception of one common component. The rough and smooth endoplasmic reticulum membranes each exhibit about 15 bands, some of which are shared

by both membranes. The outer mitochondrial membrane has three polypeptide species[30] that closely resemble three polypeptides observed in endoplasmic reticulum.

Membrane proteins of bacteria (e.g., *Mycoplasma* and *Escherichia coli*) are quite polydisperse. The envelope of *E. coli*, for example, consists of two membranes: an outer membrane containing six proteins and an inner membrane which performs the functions of the surface, inner mitochondrial, and nuclear membranes of eukaryotic cells. The inner membrane has at least 27 proteins.[31] The peptides present in the erythrocyte membrane show considerable heterogeneity.[15,32,33] As noted previously, 15 polypeptides can be detected in gel electrophoretograms, but additional polypeptides may be present in amounts beyond the level of detection using conventional staining procedures.

### *3. Chemical Composition of Membrane Proteins*

Apart from identifying peptides on the basis of molecular weight or net charge, membrane proteins when isolated from other membrane components can be further characterized on the basis of their amino acid and carbohydrate composition. Plasma membrane proteins, for example, can be classified broadly into two groups depending upon whether or not they contain covalently-bound carbohydrate. The amount and type of sugar residues varies from one membrane type to another, but most mammalian plasma membranes contain between 2 and 10% by weight of carbohydrate.

With the notable exception of Golgi membranes, most intracellular membranes contain relatively small amounts of carbohydrate. The sugars are attached to the polypeptide chain by alkali labile, O-glycosidic linkages between the side chain carbon of acetylgalactosamine, and hydroxyl groups of serine or threonine. Alternatively, alkali stable, *N*-glycosidic bonds are formed between the reducing group of acetylglucosamine and the amide group of asparagine. The sugars are usually short heterogeneous oligosaccharides ordered in specific characteristic sequences. The preponderant monosaccharide constituents of glycoproteins include galactose, glucose, mannose, fucose, *N*-acetyl-galactosamine, *N*-acetylglucosamine, and the nine-carbon amino sugar, sialic acid. Two of these, fucose and sialic acid, are usually located at the nonreducing termini of saccharide sequences.

Treatment of plasma membranes with neuraminidase to remove sialic acid residues markedly reduces the binding affinity of cationic dyes and anodic mobility in an applied potential gradient,[34] suggesting that these sugars contribute substantially to the overall surface charge of the membrane. The composition of two particular membrane glycoproteins serves to illustrate the general character of these components. Bovine rhodopsin contains around 4% sugar, one oligosaccharide unit on each 28,000 dalton polypeptide chain.[35] This heterosaccharide is composed of three mannose and three *n*-acetyl-glucosamine residues, and is covalently bound to the protein by an *N*-acetyl-glucosaminyl-aspargine linkage. The other example is the major sialoglycoprotein of human erythrocyte membrane.[36] This glycoprotein of 131 amino acids contains 64% by weight of carbohydrate and is responsible for several biological activities (the MN blood group antigen, influenza receptor activity, and phytohemagglutinin binding). A substantial proportion of the membrane carbohydrate is associated with this protein. The approximate number of residues and sugars respectively attached to each polypeptide are: 27, sialic acid; 27, galactose; 5, mannose; 4, fucose; 50, acetylglucosamine; 24, acetyl-galactosamine; 2, glucose. Three sialoglycopeptide fractions have been resolved by sodium dodecylsulphate-polyacrylamine gel electrophoresis.

Amino acid analyses have been performed on whole membrane preparations, on purified membrane proteins, and on various fragments. Studies of amino acid composition of whole membranes do not show outstanding differences among the various membranes (Table 5). The proportion of hydrophobic amino acid residues of mem-

TABLE 5

**Amino Acid Composition of Various Membrane Isolates**

| Amino acid group | Human erythrocyte membrane | Rat liver plasma membrane | | Ehrlich ascites cells | |
|---|---|---|---|---|---|
| | | Light fraction | Heavy fraction | Plasma membrane | Endoplasmic reticulum membrane |
| Basic | 12.1 | 14.4 | 12.6 | 13.6 | 13.8 |
| Acidic | 20.7 | 19.7 | 20.6 | 18.9 | 19.3 |
| Neutral | 32.5 | 35.2 | 34.2 | 33.6 | 32.3 |
| Hydrophobic | 34.8 | 31.8 | 32.7 | 34.9 | 34.0 |
| $NH_3$ | 6.9 | — | — | 14.7 | 10.8 |

*Note:* Values are expressed as moles percent. The basic amino acids include Lys, His, Arg the acidic amino acids include Asp and Glu; the neutral amino acids include Thr, Ser, Pro, Gly, Ala, 1/2 Cys; the hydrophobic residues include Val, Met, Ile, Leu, Tyr, Phe, Try.

After Steck, T. L. and Fox, C. F., in *Membrane Molecular Biology* Fox, C. F. and Keith, A. D., Eds., Sinauer Associates, Stamford, 1972, 27.

TABLE 6

**Amino Acid Composition of Soluble and Membrane Lipoproteins**

| Class | Hydrophilic mol % | Nonpolar mol % | Hydrophilic/ nonpolar ratio | Charged mol % |
|---|---|---|---|---|
| Soluble lipoproteins | 49.8 | 27.3 | 1.8 | 33.8 |
| Membrane lipoproteins | 42.7 | 29.9 | 1.4 | 29.0 |
| Soluble proteins | 49.1 | 23.9 | 2.1 | 33.3 |

After Hatch, F. T. and Bruce A. L., *Nature (London)*, 216, 1166, 1968.

brane protein fractions has been determined to see if the proteins can be distinguished from water-soluble proteins in general. The compilations of Hatch and Bruce[37] of the hydrophobic ratios of amino acids in various proteins (Table 6) indicate a higher ratio of nonpolar residues in membrane proteins, while such an increase is not apparent from the data in Table 5. However, it can be argued that a mixture of proteins has a tendency to converge toward a common pattern which is unlike that of the individual components; Rosenberg and Guidotti[38] showed that the amino acid profiles of unfractionated proteins from various membrane sources resemble that of the total soluble cytoplasmic fraction of *E. coli*. As we have already indicated, membrane preparations sometimes contain contaminations from nonmembrane proteins (for example, adsorbed and entrapped soluble protein). Another complication is that even the classification of amino acids as polar or hydrophobic can be contentious.[39-41] The classification of amino acids shown in Table 5 is based upon the studies of Nozaki and Tanford[39] in which the free energy of transfer of amino acid side chains from water to less polar solvents was examined. Their quantitative hydrophobicity scale allows each side chain to be weighted according to its free energy of transfer, as wide differences exist among them.

A comparison of the amino acid composition of intrinsic and extrinsic membrane proteins indicates that the proportion of polar to more hydrophobic amino acids is, in general, unrelated to the location of the protein in the membrane (see Table 7).

TABLE 7

**Amino Acid Composition of Elutable and Nonelutable Membrane Proteins**

| | Elutable | | | Nonelutable | | | |
|---|---|---|---|---|---|---|---|
| Amino acid group | Human erythrocyte membrane | Liver Eigen protein | *S. Faecalis* ATPase | Bovine $F_1$ ATPase | Erythrocyte sialoglyco-protein | Bovine retinal rhodopsin | Bovine myelin pro-teolipid |
| Basic | 15.1 | 16 | 12.3 | 13.7 | 11.4 | 8.6 | 9.4 |
| Acidic | 31.4 | 31 | 23.0 | 19.6 | 18.4 | 15.3 | 9.9 |
| Neutral | 25.3 | 24 | 34.3 | 36.2 | 34.3 | 35.2 | 41.6 |
| Hydrophobic | 27.8 | 29 | 31.0 | 30.3 | 36.8 | 40.8 | 39.7 |

*Note:* Values are expressed as moles percent. The basic amino acids include Lys, His, Arg; the acidic amino acids include Asp and Glu; the neutral amino acids include Thr, Ser, Pro, Gly, Ala, 1/2 Cys; the hydrophobic residues include Val, Met, Ile, Leu, Tyr, Phe, Try.

After Steck, T. L. and Fox, C. F. in *Membrane Molecular Biology*, Fox, C. F. and Keith, A. D., Eds., Sinauer Associates, Stamford, 1972, 27.

TABLE 8

**Amino Acid Composition of Membrane Proteins Classified According to the Physical Properties of the Constituent Amino Acids (mol %)**

| Amino acid group | Erythrocyte glyco-protein | Bovine rhodopsin | Halobacterium rhodopsin | Nonneutral proteolipid | Myelin proteolipid | Sarcoplasmic ATPase | *S. aureus* phosphokinase |
|---|---|---|---|---|---|---|---|
| Basic | 11.46 | 8.51 | 7.44 | 7.22 | 9.34 | 11.00 | 10.4 |
| Acidic and polar | 32.01 | 32.33 | 30.99 | 28.08 | 29.55 | 34.19 | 23.4 |
| Nonpolar | 49.06 | 52.34 | 51.66 | 55.45 | 50.67 | 47.34 | 60.4 |

After Guidotti, G., *Annu. Rev. Biochem.*, 41, 731, 1972.

Indeed, by this criteria alone one would expect glycoproteins, for example, sialoglycoprotein of the erythrocyte membrane, to be released from membranes under relatively milder conditions than proteins with considerably less carbohydrate, for example, spectrin, due to the preponderance of polar sugar residues. This feature can be explained by the heterogeneous distribution of polar and less polar amino acid residues along the polypeptide chain. Thus amino acid analyses of peptide fragments derived from tryptic digestion of membrane glycoproteins show these to consist of a high proportion of polar amino acids while other regions of the polypeptide chain, containing less polar amino acids, are protected against hydrolysis because of their location within the hydrophobic region of the membrane (see Section IV.B.2).

In some cases, the conditions required to extract proteins from particular membranes is reflected in the amino acid composition of the protein. Myelin from the central nervous system, for example, contains three major polypeptides. One has been called the basic protein because it can be extracted with weak acids and because a large fraction of its constitutent amino acids are basic ones. The molecular weight of this protein is about 18,000, and the amino acid sequence of the protein from bovine[42] and human[43] has been determined. The second major protein component of myelin is the proteo-lipid[44] which is soluble in 2:1 (by volume) chloroform-methanol. It has a unique amino acid composition in which the nonpolar amino acids account for approximately 50% or more of all the amino acids on a molar basis.[45-47] As pointed out by Hatch and Bruce,[37] an amino acid composition of this type is unusual and is probably found in only a few membrane proteins (Table 8). The molecular weight is about 25,000. The remaining 20% of the protein present in myelin is called Wolfram protein, which is soluble in acidified chloroform-methanol.[48]

When the complete amino acid composition of a membrane protein has been determined, the homogeneity and size of the polypeptide can be checked by peptide mapping.[49] After tryptic digestion, the peptides are separated by chromatographic and electrophoretic methods and counted. For homogeneous proteins the number of peptides equals n + 1 where n is the number of lysine plus arginine residues. When the extracted protein is contaminated with other membrane proteins, more peptides will be produced than would be predicted from the amino acid analysis and correspondingly less of each peptide than expected for an equivalent amount of starting material. Some caution must be exercised when interpreting peptide maps in this way since a number of membrane proteins have been shown to yield homologous peptides on trypic digestion.

End-group analysis in which the C- and N-terminal amino acids are reacted covalently with specific reagents has only limited application in the case of membrane pro-

TABLE 9

**Marker Enzymes Conventionally Used to Identify Rat Liver Membranes or Organelles**

| Membrane | Enzyme | Enzyme commission number | Number of different enzyme activities reported |
|---|---|---|---|
| Plasma membrane | 5′ Nucleotidase | 3.1.3.5. | 24 |
| Endoplasmic reticulum | Glucose 6 phosphatase | 3.1.3.9. | 75. |
| Smooth surface | Uridine diphosphatase | 3.6.1.6 | |
| Rough surface | Various esterases | 3.1.1.1. | |
| Mitochondria | | | 70 |
| Inner membrane | Succinate dehydrogenase | 1.3.99.1 | 21* |
| Outer membrane | Kynurenine hydroxylase | 1.14.1.2 | 13* |
| Golgi complex | UDP-galactose:N-acetylglucosamine galactosyltransferase | 2.4.1.22(A) | |
| Lysosomal enzymes | Acid phosphatase and other hydrolytic enzymes | | 14 |
| Peroxisomal enzymes | D-amino acid oxidase | 1.4.3.3 | |

* Nine enzymes are common to inner and outer membranes.

teins. This is because the N-terminal amino groups and/or C-terminal carboxyls are not free to react and have been found to be blocked in several purified membrane polypeptides. Such lack of N- and/or C-terminal reactivity has been observed with bovine rhodopsin,[50] two subunits of the ATPase of *Streptococcus faecalis*,[51] and the major sialoglycoprotein of the human erythrocyte membrane.[36]

### *4. Membrane-Bound Enzymes and Antigens*

Cell membranes not only function as simple barriers to the movement of substances between the cell and its environment and between membranous compartments within the cell but they serve as a support for a wide variety of enzymes. Nearly all biological membranes exhibit at least one form of enzyme activity. A number of these membrane-bound enzymes are associated with only one type of membrane, and the localization by cytohistochemical techniques or assay of activity provides a useful biochemical marker for these particular membranes. Furthermore, when membranes are isolated from tissue homogenates, the assay of enzymes associated exclusively with each subcellular membrane can be used to establish the purity of membrane fractions and the extent of contamination with other membrane fractions. Some enzyme activities conventionally used for identification of subcellular membranes of rat liver are presented in Table 9. The last column of this table enumerates the different type of enzyme activity reported for each particular membrane. Clearly the protein composition of each membrane is extremely complex when all the enzymes that are associated with the membrane are taken into account and is comparable with the diversity that is encountered with the plasma proteins or the cytoplasmic proteins. Nevertheless, it should be emphasized that the contribution of many of these proteins to the total membrane mass is relatively small. For example, estimates of the number of $Na^+$-$K^+$-dependent ATPase sites per human erythrocyte range around 200.[52] It has been estimated that there are approximately five million polypeptide chains per red cell membrane, so that functional proteins such as $Na^+$-$K^+$-ATPase constitute only a minute portion of the total. This is in contrast to membranes such as the sarcoplasmic reticulum and retinal rod outer segment disc membranes where $Ca^{2+}$-ATPase and rhodopsin, respectively, represent the major protein components.

Apart from enzyme and transport functions, membrane proteins and glycoproteins

exhibit important antigenic activities. These proteins may constitute a significant proportion of the total membrane protein. Sialoglycoprotein, responsible for MN antigenicity of the human erythrocyte membrane, for example, constitutes nearly 3% of the total membrane protein or approximately 500,000 molecules per cell.[33] In the case of rat liver membranes, each particular membrane has been shown to give rise to unique immunological specificities, which has been exploited to identify each membrane. Thus distinctive and unique autogenic determinants have been established for nuclear membranes, endoplasmic reticulum, inner and outer mitochondrial membranes, and lysosomal membranes all of which are different from plasma membrane, and none of these antigens were found to cross-react with other membranes.[52]

Other antigens located on the surface of all mammalian cells appear to function in cell-cell recognition and play a role in membrane reactions such as density-dependent inhibition of growth and mitogenesis. The recognition or histocompatibility antigens are involved in allograft phenomena, and the antigenic specificities have been investigated in the mouse and humans.[53] In the case of human histocompatibility antigens (HL-A), more than 20 immunologically distinct specificities have been identified, all of which arise from different gene combinations although many of these are only weakly expressed and difficult to detect by standard immunological tests.

*5. Protein Primary and Secondary Structure*

If one begins with the premise that the primary structure of a protein predetermines the more complex levels of organization of the molecule, then it should be possible, if the amino acid sequence of a protein or peptide is known, to predict at least the secondary structure most likely for that sequence. Two approaches have been developed to relate the primary amino acid sequence to conformation: a statistical[54, 55] and a stereochemical method.[56, 57] Schultz et al.[58] have successfully applied a statistical analysis of the amino acid distribution among regions of globular proteins in different conformation to predict the structure of the proteins. Such proteins are soluble in water and can be made to form crystals so their structure can be readily and accurately verified by spectroscopic and X-ray techniques. The amino acid sequence of several membrane proteins or peptides are now known. One of the best examples is that of cytochrome c for which a complete amino acid sequence determination has been performed and accurate and detailed structure assigned. As one might expect from the location of this protein on the outer surface of the inner mitochondrial membrane, the structure is not dissimilar to that of other heme proteins found in free solution. Apart from cytochrome c there is little information concerning amino acid sequence of other membrane proteins.

Data of five membrane-associated proteins are presented in Table 10 and include three coat proteins of filamentous bacteriophages, a protein from the outer envelope of *Escherichia coli* and the hydrophobic peptide derived from erythrocyte glycophorin. X-ray diffraction[64] and spectroscopic analyses[65,66] of the three coat proteins indicate that they are substantially $\alpha$-helical in structure. The outer envelope protein of *E. coli* is believed to form polar channels across the membrane[67] and after extraction is largely helical in conformation. The hydrophobic peptide of erythrocyte glycophorin is thought to be the region of the polypeptide chain that traverses the lipid bilayer.[68] It is helical in trifluoroethanol, and the helix is more resistant to disruption by trifluoroacetic acid than is that of poly-L-alanine of the same chain length.[69] Green and Flanagan[70] have attempted to predict the conformation of these membrane-associated polypeptides from the sequence of amino acids and found that stereochemical rather than statistical methods provide a more accurate estimate of the secondary structure of these proteins.

One of the major problems in determining the structure of membrane proteins is

TABLE 10

**The Content of Hydrophobic Amino Acid Residues and Secondary Structure of Some Membrane-Associated Proteins and Peptides**

| Peptide | % Hydrophobic residues | % α helix | Ref. |
|---|---|---|---|
| Pfl coat protein | 56.5 | 90 | 59,64 |
| fd coat protein | 56.0 | 90 | 60,66 |
| zj-z coat protein | 58.0 | 70* | 61,70 |
| *E. coli* envelope protein | 36.8 | 70 | 62,63 |
| Glycophorin hydrophobic peptide | 54.9 | 100 | 68,69 |

* Calculated according to Lim.[56,57]

the fact that the environment of most of these proteins differs from two extremes. Some proteins are known to be located almost exclusively in a polar environment while others are interpolated completely into the hydrophobic region of the membrane. The majority of membrane proteins, however, are likely to possess regions of the polypeptide chain in water while other sequences may reside in the lipid hydrocarbon chain region, and the proportion of the chain in the respective environments varies widely between different membrane proteins. The effect of the surrounding solvent molecules on water-soluble proteins is well documented, but in a hydrophobic environment the folding of the polypeptide may deviate considerably from the type of arrangement that one encounters in cytoplasmic proteins. There have been attempts to define the influence of solvent polarity on protein conformation, and it has been shown that solvents of low dielectric, for example, favor α-helix and β-sheet but, in general, α-helix appears to be the more stable conformation in organic solvents. Polytyrosine is known to form an aggregated antiparallel β-sheet in water, but on addition of ethanol the polypeptide is converted to a more α-helical structure.[73]

### *6. Detailed Structure of Proteins in Membranes*

Direct examination of the structure of proteins by X-ray crystallography techniques is largely precluded in the case of most membrane proteins because isolation of these proteins from other membrane constituents often leads directly to changes in conformation. Furthermore, it is extremely difficult to prepare crystals of such material suitable for X-ray analysis. A successful method for direct examination of membrane proteins *in situ* is electron diffraction.[74] The technique consists of taking electron micrographs at electron doses which are not completely destructive and compensating for the poor contrast by averaging over a large number of unit cells in an ordered arrangement of the specimen. The method has been applied to study the structure of bacteriorhodopsin, which is located in a specialized region of the membrane of *Halobacterium halobium* referred to as the purple membrane[75] and the arrangement of cytochrome oxidase molecules extracted from mitochondria with detergent and reconstituted into lipid vesicles[76] (see section V.C.3).

Infrared and ultraviolet spectroscopic techniques have been used to examine the structure of proteins in membranes.[77] One of the inherent problems in measurements of this type is that the spectral properties reflect the average conformation of what may be a rather diverse group of molecules, and consequently the structure of individual components cannot be readily evaluated. Moreover, each protein contributes to the average conformation to an extent depending on the relative amount of that protein in the structure. A further complication in the case of optical measurements of suspensions of membranes is that the turbid nature of these preparations produces marked

distortions of the spectral features of interest, and elaborate corrections are necessary to derive reliable information on protein structures.

The conformation of proteins in erythrocyte membranes and myelin has been investigated by infrared spectroscopy.[78,79] Spectra obtained from completely or partially hydrated membranes show strong symmetrical amide I bands about 1652 $cm^{-1}$ characteristic of $\alpha$-helix or random coil arrangements, but virtually no trace of a peak at 1630 $cm^{-1}$ to indicate the presence of $\beta$-conformation. The problem of estimating the relative proportions of $\alpha$-helix and random coil configurations has been examined by Steim[80] who found a shift in the amide I band from 1651 $cm^{-1}$ to about 1640 $cm^{-1}$ and a corresponding decrease in the amide II band on transferring membranes from $H_2O$ to $D_2O$. These changes are consistent with the presence of a considerable amount of disordered structure in erythrocyte membranes with the majority of the chains freely accessible to water. An attempt to demonstrate that membrane proteins are not unique in that they can be converted to $\beta$-structure by thermal denaturation[78] showed no differences in characteristic amide I or amide II bands in membrane preparations maintained at temperatures ranging from −150° to 150°C. When the residue remaining after complete extraction of the lipids with organic solvents was examined, a broad amide I band was observed centered about 1652 $cm^{-1}$, with a pronounced shoulder at 1628 $cm^{-1}$ characteristic of $\beta$-conformation. Moreover, unless all traces of lipid were removed by the extraction procedure, a structural transition from $\alpha$-helix to $\beta$-conformation could not be demonstrated, even by heating to 150°C, suggesting that residual lipids that remain tightly bound to the protein exert a stabilizing effect on the structure. The absence of appreciable $\beta$-structure has also been reported in a number of other membrane preparations, with the notable exception of the inner mitochondrial membrane in which the infrared spectrum indicates a mixture of $\beta$-conformation and random coil.

Protein structure has been examined by optical rotatory dispersion and circular dichroism methods in plasma membrane from a variety of cell types as well as preparations of endoplasmic reticulum and mitochondrial membranes. The general conclusions from such studies is that membrane proteins show $\alpha$-helical conformations but absolute amounts of $\alpha$-helix, $\beta$-structure, or random coil cannot be derived with any great precision. On the assumption that no appreciable $\beta$-conformation exists in membrane proteins, semiquantitative estimates of $\alpha$-helix content usually vary between 30 and 50%, although values as high as 60% and as low as 10% have been reported. The validity of the underlying assumption used in these calculations cannot be assessed from spectral considerations alone because a considerable amount of $\beta$-conformation may be present without appearing obvious from the spectrum. Infrared spectroscopy must be employed independently to confirm the absence of $\beta$-conformation. Urry[81] has performed a careful study of the C.D. spectra of a number of membranes. He has applied corrections for decreased amplitude and spectral broadening and derived values of ellipticity at 192 nm and 224 nm. These values in five different membranes, together with the molar absorption coefficient at 192 nm (Table 11), indicate the presence of more helical structure in erythrocyte, mitochondrial and plasma membranes compared with sarcoplasmic reticulum and axonal membranes where the proteins appear to be in a more extended conformation.

### C. Summary and Critique

As a prelude to the detailed analysis of biomembranes, the structures must be isolated from the intact cells or tissue in reasonable yield and in undamaged condition. Detailed procedures have been devised to destroy the integrity of intact biological systems and allow separation of component membranes. The use of appropriate homogenization methods is essential to avoid both mechanical damage and/or digestion, or

TABLE 11

**Circular Dichroism Parameters and Extinction Coefficients of Some Mammalian Membrane Preparations**

| Membrane | Ellipticity [θ] × $10^{-4}$ 192 nm | Ellipticity [θ] × $10^{-4}$ 224 nm | Molar absorption coefficient (× $10^{-3}$) 192 nm |
|---|---|---|---|
| Human erythrocyte | 3.37 | 1.70 | 8.7 |
| Beef heart mitochondria | 3.22 | 1.65 | 7.6 |
| Rat liver plasma membrane | 2.92 | 1.56 | 9.0 |
| Sarcoplasmic reticulum | 1.87 | 1.08 | 7.5 |
| Axonal membranes | 1.13 | 0.89 | 12.6 |

Data from Urry, D. N., *Ann. N.Y. Acad. Sci.*, 195, 108, 1972

disruption caused by release of enzymes from organelles such as lysosomes. Furthermore, vesiculation of membrane structures by vigorous methods leads to contamination of one morphologically distinct membrane fraction with another. Apart from the commonly used differential sedimentation method of separating membrane fractions, new methods giving high yields including zonal centrifugation and affinity chromatography will be further refined. It may be anticipated that the latter method will be more generally applied as immunological methods advance allowing the isolation of pure membrane fractions in high yield.

The techniques described for extraction of lipid and protein components and the methods available for their detailed analysis continue to proliferate. Undoubtedly techniques such as gas chromatography-mass spectrometry, gel filtration, and polyacrylamide gel electrophoresis have contributed greatly to our knowledge of the composition of membranes, and refinements are from time to time introduced. Recent developments with two-dimensional gel electrophoresis of proteins, for example, will permit the resolution of nearly all the proteins present, even in highly complex membranes.

When we consider the composition of different biological membranes, a number of important questions arise for which we as yet have no satisfactory answers. Why, for example, is such a complex distribution of lipids, including phospholipids and their associated fatty acids differing in both chain length and number of unsaturated bonds, present in biological membranes? Does each molecular species perform separate functions or simply create the appropriate physical environment for the protein component? Why is cholesterol found in some cell membranes (or other sterols in microorganisms) but not in significant amounts in others? What is the function of charged phospholipids and their relationship to the zwitterionic phospholipids? We have some knowledge of the complexity of the sugar residues of glycolipids and glycoproteins, but the role of these components remains largely obscure. With regard to the protein components, it seems clear that they are not required to sustain the structure of cell membranes, as was once believed by some workers, and many are enzymes which are involved in well-characterized biochemical reactions. Nevertheless, the function of many membrane proteins is unknown. Although the structure of a few selected membrane proteins are known in some detail, virtually nothing is known about the structure of the vast majority of these proteins at any level of organization. Moreover, the significance of the hydrophobic-hydrophilic amino acid ratios of membrane proteins is not yet appreciated in the context of the theories that pertain to the forces involved in higher orders of membrane protein structure. This results mainly from our ignorance of the molecular environment in which different regions of the polypeptide chain exists.

# III. PHYSICAL PROPERTIES OF LIPIDS IN AQUEOUS SYSTEMS

## A. Water in Phospholipid Systems

Phospholipids exhibit interesting behavior in the presence of water. As with their thermotropic mesomorphism, where they do not pass directly from the crystalline state to a liquid, the phospholipids in general do not pass directly from the crystalline state to a solution in the presence of water. Various hydrated phases are encountered before solution of the phospholipids in water occurs. Such behavior is referred to as lyotropic mesomorphism. The lyotropic phases exhibit thermotropic mesomorphism; in other words, the particular phase obtained is a function both of water content and of temperature.

The importance of the thermotropic phase transition temperature can be seen when we appreciate that when water diffuses into the lattice it does so into the polar (ionic) region only when the temperature is reached at which the hydrocarbon chains "melt". If the temperature is higher than this, there is a simultaneous dissociation of the ionic lattice by the penetration of water and melting of the hydrocarbon chain region. The temperature of transition depends upon the nature of the hydrocarbon chains and of the polar region of the molecule, the amount of water present, and the presence of any solutes dissolved in the water.

Once the water has penetrated into the lattice of the amphiphile and the sample is then cooled to below the transition temperature, the hydrocarbon chains rearrange themselves into an orderly crystalline lattice, but the water is not necessarily expelled from the system. These phases containing crystalline paraffin chain regions are called gels; these gels may or may not be metastable. If they are metastable, they transform over a period of time to a suspension of microcrystals of the amphiphile in water, the cogel state. The cogel is a stable phase; its structure is independent of the thermal history of the sample.

Bear, Palmer, and Schmitt[82] and Palmer and Schmitt[83] were the first to study, by X-ray diffraction, the lyotropic phases obtained by mixing natural lipids with water. Among the nerve lipid fractions studied was lecithin, which, on addition of two parts of water to one of lecithin, gives rise to a phase with a lamellar structure possessing liquid-like hydrocarbon chains and a long spacing of 6.9 nm some 60% greater than that of the dry material. For over a quarter of a century no further work was published concerning either the structure or the range of existence of lecithin-water phases. In 1967 three papers by independent groups were published. Two of the papers were concerned with lecithin extracted from egg yolk;[84,85] the other covered a homologous series of 1,2-diacyl-L-phosphatidycholines (lecithins).[86]

### *1. Lipid Phase Diagrams*

It can be seen from the phase diagram of the 1,2-dipalmitoyl-L-phosphatidylcholine-water system (Figure 2), that on addition of water, the transition temperature, T of the phospholipid is lowered to a limiting value $T_c$. The effect of water on 1,2-distearoyl-L-phosphatidylcholine has been studied by differential scanning calorimetry.[87] The anhydrous lipid undergoes an endothermic phase transition, but the transition tends to be noncooperative and occurs at a much higher temperature (85°C) than when the lipid is dispersed in excess water. In this case the transition becomes sharper indicating cooperative effects, and the temperature of transition decreases to 53°C. Similar behavior has been noted with 1,2-dimyristoyl-L-phosphatidylcholine (Figure 3). This transition temperature, $T_c$, is the minimum temperature required for water to penetrate between the layers of the lipid molecules. Above the $T_c$ line the phosphatidylcholine-water system exists in a mesomorphic lamellar phase, in which the hydrocarbon chains are in a liquid-like state. The composition of the system at maximum hydration is

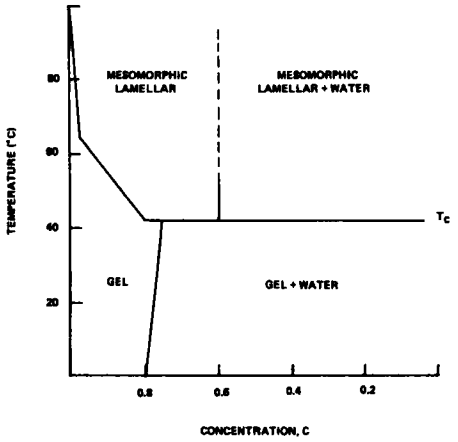



FIGURE 2. Phase diagram of 1,2-dipalmitoyl-L-phosphatidylcholine-water system. The $T_c$ line distinguishes the mesomorphic lamellar phase from the gel. The transition temperature reaches a minimun of 41°C when the concentration of water exceeds about 25% by weight. Both the mesomorphic lamellar and gel phases are stable in excess water.

about 40 wt % water. Addition of more than 50 wt % water gives rise to a two-phase system consisting of fragments of the lamellar phase at maximum hydration dispersed in the excess water.

When the phosphatidylcholine-water system cooled below the $T_c$ line, the hydrocarbon chains adopt an ordered packing. The structure of this phase, the gel, is lamellar with the hydrocarbon chains packed in a hexagonal subcell with the chain axes inclined at 58°C to the lipid-water interface. Lipid bilayer structures in which the hydrocarbon chains are in vertical, "melted", and tilted arrangements are illustrated in Figure 4.

The phase diagrams of the different chain length lecithin-water systems are essentially equivalent and are disposed along the temperature axis according to the melting temperature ($T_c$) of the hydrocarbon chains. It is noteworthy that in the range of water concentrations $1.0 > C > 0.8$, which represents an appreciable amount of water in the system, no transition is observed in the heating or cooling curves due to any melting of ice or freezing of the water present. When the water content is greater than 20% ($c < 0.8$) the lipid endothermic transition temperature ($T_c$) remains constant, and a peak at 0°C, due to the melting of ice, can now be observed in the heating curve. As the concentration of water in the mixture further increases, so does the size of this peak. Quantitative studies are interpreted as showing that a proportion of the water is bound to the lecithin in a fixed ratio of 1:4 by weight (10 mol water/mole lecithin). This bound water is due to the formation of a hydrate structure associated with the polar group. The amount of bound water is independent of the fatty acid composition of the phospholipid, but is dependent upon the nature of the hydrophilic group. Bound water associated with the phosphatidylethanolamines and sodium phosphatidylserines has been reported.[90]

All the different lecithins studied behave similarly, and in Figure 5 the variation of $T_c$ with chain length is shown. The heat absorbed at $T_c$ for lecithin water systems with $c < 0.7$ (Table 12) is seen to be dependent on the hydrocarbon chain length. For the series of fully saturated lecithins there is a difference of about 10 kJ $mol^{-1}$ for a chain-length difference of two methylene groups. Extrapolation to zero at this transition

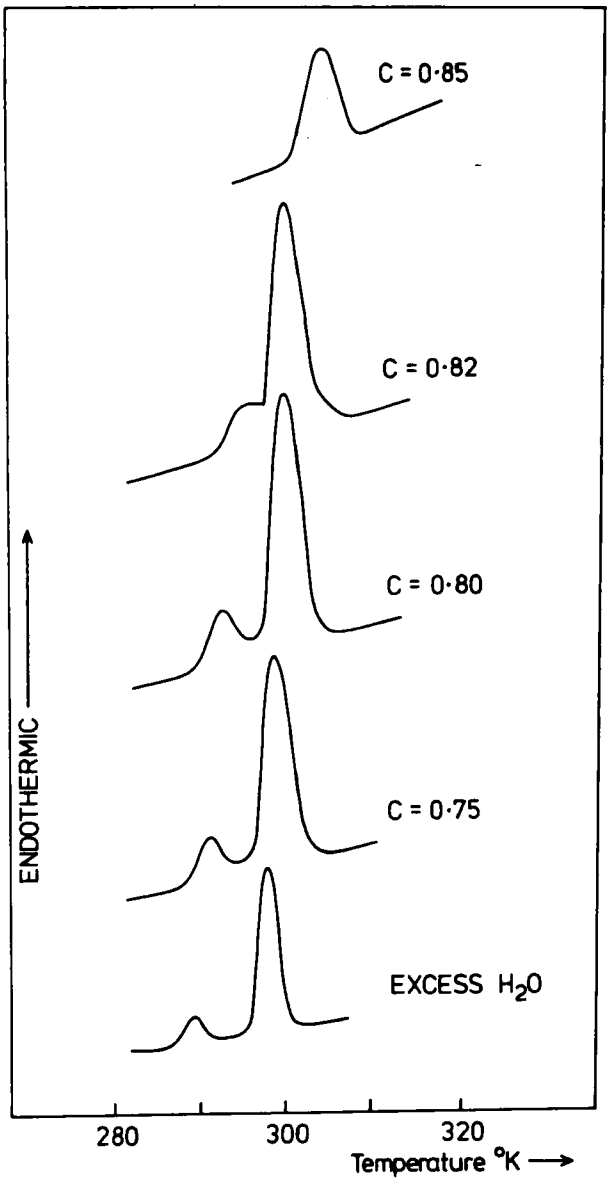



FIGURE 3. Differential scanning calorimetric heating curves of 1,2-dimyristoly-L-phosphatidylcholine dispersed in increasing amounts of water. (C) represents the weight of lipid per unit weight of water. The lipid containing only 15% by weight of water is seen to undergo a nonco-operative phase transition at about 32°C, and no pretransition endotherm is observed. The endotherm becomes sharper with increasing water content reaching a lower limiting value, $T_c$ of about 23°C in excess water. There is also a corresponding decrease in the temperature of the pretransition endotherm. (Data from References 88 and 89.)

shows that the $C_7$ and shorter chain-length lecithins have a negative heat of transition. A small so-called "pretransition" peak is observed when $c < 0.8$. The temperature interval between this peak and the main endothermic peak ($T^*_c$) increases (see Figure 5) as the chain length of the lecithin becomes shorter. This pretransition peak has been

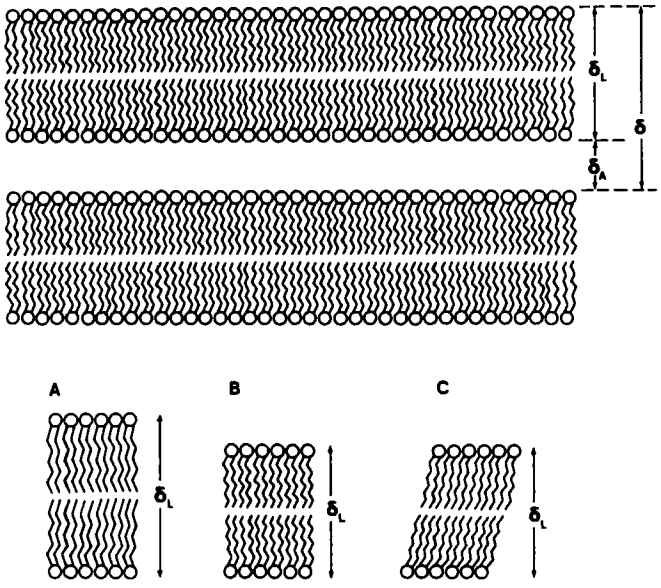



FIGURE 4. Lamellar lipid bilayer structures that spontaneously form in water. The repeat distance, $\delta$, of concentric bilayers of multilamellar forms of polar lipids in water is a composite of the bilayer thickness, $\delta_L$, and the aqueous region between successive bilayers, $\delta_A$. The distance $\delta_A$ depends on the presence of charged groups on the lipid molecules and the nature and concentration of ions in the aqueous phase. The hydrocarbon chain organization determines the bilayer thickness, $\delta_L$. The hydrocarbon chain organizations in vertical or $\beta$(A) "melted" (B) and tilted or $\beta'$ arrangements (C) are illustrated.

shown to be associated with a change of polar group organization which accompanies a change of tilt of the liquid chains from a condition of being tilted to the plane of the lamellae to the vertical position.[93] Pure lecithin systems appear sensitive to foreign molecules, e.g., cholesterol,[92] drug molecules, and other lipids. Small quantities present in the lamellae cause the chains to move to the vertical condition[94] (see below).

A complete binary phase diagram of the egg-yolk lecithin-water system has been described by Small[85] which is essentially the same as those found for the lecithins of discrete chain lengths except that the $T_c$ line is rather broad and ill-defined. This observation is consistent with other work on phospholipids derived from a heterogeneous mixture of fatty acids.

Various structures that can exist below the thermotropic phase-transition temperature have been described.[95] A $\beta$ form is obtained with heterogeneous lipids and is characterized by an orientation of the lipid chains vertical to the plane of the lamellae (see Figure 4). Another structure designated $\beta'$ is the form observed with pure lipids of identical chains but which have the chains tilted with respect to the normal in relation to the lamellae. Both forms are packed with rotational disorder in a two-dimensional hexagonal lattice. We have already noted that upon heating pure lecithins such as dipalmitoyl lecithin, a transformation from the $\beta'$ to the $\beta$-form takes place just prior to the fluid condition. (The fluid form is termed the $\alpha$-form). The use of this $\beta$ and $\alpha$ convention in this way is somewhat unsatisfactory because with many long-chain molecules the $\alpha$-form refers to the form having hexagonally packed chains while the $\beta$-form refers to the forms having orthorhombic and triclinic packed chains.[96] Tardieu et al.[95] also found that as water is added to anhydrous preparations of pure dipalmitoyl lecithin, the thickness of lipid leaflet decreases as the amount of water increases. This

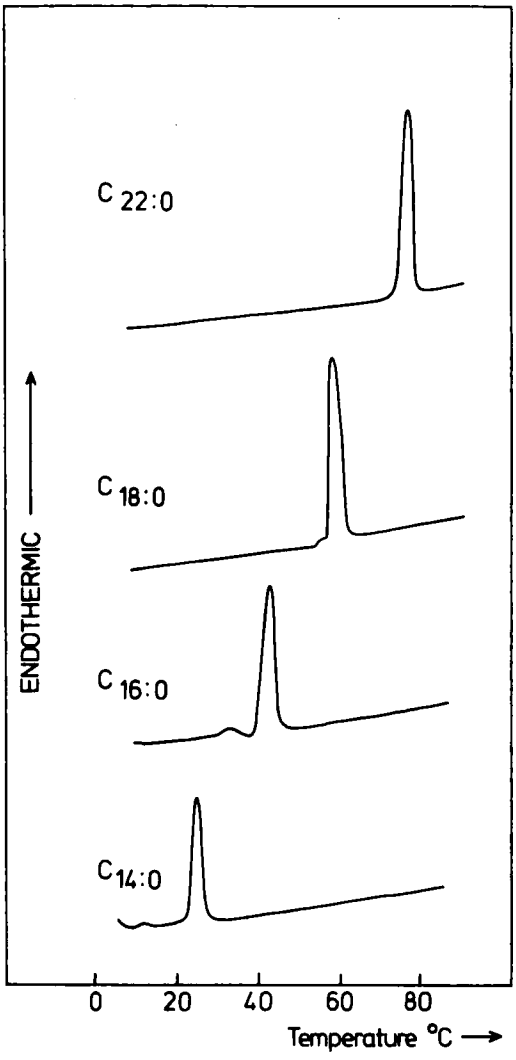



FIGURE 5. Differential scanning calorimetric heating curves of a homologous series of fully saturated 1,2-diacylphosphatidylcholines ranging in chain length from $C_{14}$ to $C_{22}$. Each phospholipid was dispersed in an equal weight of water. As the length of the hydrocarbon chains increase, the main endothermic transition, $T_c$, increases from about 23° ($C_{14}$), 41° ($C_{16}$), 56° ($C_{18}$), and 76° ($C_{22}$). The corresponding heats of transition also increase with increasing chain length from 41 $Jg^{-1}$ ($C_{14}$), 50 $Jg^{-1}$ ($C_{16}$) to 57 $Jg^{-1}$ ($C_{18}$). The temperature of the pretransition endotherm increases relative to the main endothermic transition temperature as the chain length is increased and is coincident with $T_c$ for the $C_{22}$ phospholipid. (Data from References 91 and 92.)

was believed to be associated with an increased tilt of the chains with respect to the lamellae.

*2. Physical Studies of Phospholipid-Water Systems*

The lecithin-water system has received much attention because these lipids are prom-

TABLE 12

**Heats and Temperatures of Transition for 1,2-Diacyl-L-Phosphatidylcholine-Water Systems**

| 1,2-diacyl phosphatidyl-choline | Limiting transition temperature ($T_c$) (°C) | Heat absorbed at $T_c$ (kJ mole$^{-1}$) phospho-lipid | Entropy change at $T_c$ (J mole$^{-1}$) | Temperature of "pretransition" peak (°C) |
|---|---|---|---|---|
| Dibehenoyl | 75 | 72.0 | 205 | 75 |
| Distearoyl | 58 | 51.8 | 156 | 56 |
| Dipalmitoyl | 41 | 41.5 | 122 | 35 |
| Dimyristoyl | 23 | 31.9 | 108 | 14 |
| Dilauroyl | 0 (under the ice peak) | — | — | — |
| Dioleoyl | −22 | 36.5 | 154 | — |
| Egg yolk* | −15/−7 | — | — | — |

* The egg-yolk lecithin transition is broad in water due to the heterogeneous fatty acid composition. The lower temperature is from the heating curve, the higher from the cooling curve.

Data from Chapman, D., *Biological Membranes*, Vol. 2, Chapman, D. and Wallach, D. F. H., Eds., Academic Press, London, 1973, 91.

inent constitutents of animal cell membranes. A range of physical techniques have now been applied to the study of these systems including nuclear magnetic resonance (NMR) spectroscopy. A study of the lipid phase transition using wide line NMR spectroscopy showed that a marked line-narrowing associated with the anisotropic but rapid molecular motion of the acyl-chain protons takes place at the transition temperature.[97] Also, just prior to the transition temperature a line narrows which is associated with the rapid molecular motion of the $-N(CH_3)_3$ groups. This study also showed that at temperatures less than the phase transition temperature there is restricted rotational and oscillatory modes of reorientation with a line width considerably reduced from the rigid lattice value.

A number of other NMR studies have been made of lipids through the lipid transition temperature[98] including high resolution proton NMR.[99] The $^1H$ NMR spectrum of the dipalmitoyl lecithin-water system at different temperatures is shown in Figure 6. The signal associated with the $-N(CH_3)_3$ group can be seen to appear prior to the main transition. Deuterated lipids have been examined by deuteron magnetic resonance methods[100] and calculations made to obtain the order parameter along the hydrocarbon chains.[101,102] Other studies of $^{13}C$ nuclear magnetic resonance of dipalmitoyl and egg lecithins have also been reported.[103,104] Spin lattice relaxation times of $^{13}C$ egg lecithin compared with those of specifically deuterated stearic acid probes interpolated into bilayers of the lecithin are presented in Table 13. The order parameter derived from the latter measurements is considered in the following section.

Electron spin resonance spectroscopy using spin labels has also been used to study the lecithin-water phase transition,[108-110] and some possible difficulties associated with monitoring phase transitions using the spin-label technique have been discussed.[111] $^{31}P$ NMR has also been applied to a study of the head group mobility through the phase transition region.[112,113]

Water organization associated with the lipid molecules has been studied using deuteron magnetic resonance. When dipalmitoyl lecithin is heated through the phase transition temperature, the splitting of the deuteron doublet decreases sharply upon heating to the transition temperature. The splitting then increases with further increase in temperature. It has been suggested that these effects are probably due to the increasing mobility of the polar group with increasing temperature up to $T_c$.[114] The further in-

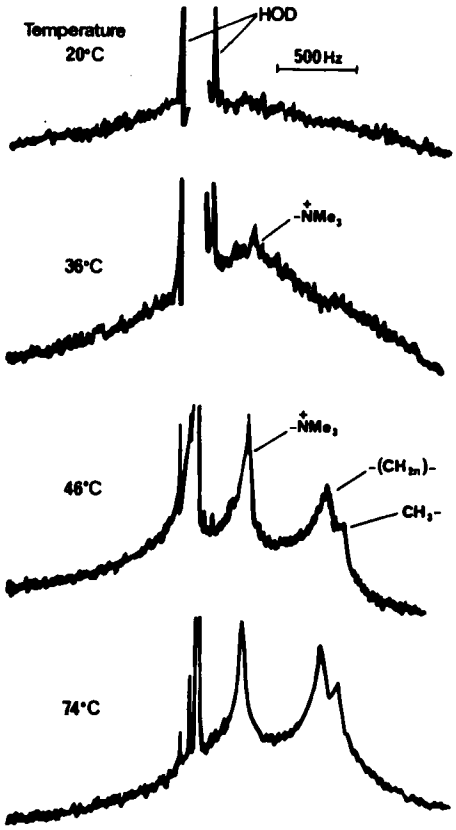



FIGURE 6. High resolution proton magnetic resonance spectra of 1,2-dipalmitoyl-L-phosphatidylcholine dispersed in $D_2O$. As the temperature is increased from 20° to 36° (below $T_c$), resonances from the -$\overset{+}{N}$Me protons can be resolved. Heating above $T_c$ to 46°C considerably increases the -$\overset{+}{N}$Me proton signal and resonances of the methylene and terminal methyl protons of the hydrocarbon chains can be observed. A further increase in temperature to 74°C sharpens each of the respective resonances. The data was derived from Reference 99 in which a 200 MHz spectrometer was used.

crease in splitting may be associated with a redistribution of the bound water. As the head groups move apart, binding sites are exposed which cause more restriction of the hydrating water molecules.[115]

Fluorescence studies,[116,117] triplet probes,[118] freeze-fracture electron microscopy,[119] laser Raman spectroscopy,[120,121] light scattering[122,123], and dilatometry[124] are among the many techniques applied to the study of phase transitions of lipid-water systems. Triplet probes have been used to estimate the change in diffusion coefficient of these triplet-labeled lipids above and below the transition temperature.[118]

A variety of other lipid-water systems have been examined.[84] These include egg-yolk phosphatidylethanolamine, sphingomyelin, egg-yolk lysolecithin, a human brain extract, a mitochondrial lipid extract,[125] and an erythrocyte lipid extract.[126] Luzzati[127] has pointed out several other lipid water forms that can exist with these systems, e.g., hexagonal as well as lamellar phases. The mitochondrial lipid system is of particular

TABLE 13

**Spin-Lattice Relaxation Times ($T_1$) of $^{13}C$ of Egg Lecithin and Specifically Deuterated Stearic Acid Interpolated Into Egg Lecithin Bilayer Vesicles**

| Hydrocarbon chain segment | | $T_1$ (sec) at 30° | |
|---|---|---|---|
| | | $^{13}C$[a] | $^{2}H$[b] |
| 18 | $-CH_3$ | 2.8 | 0.32 |
| 17 | $-CH_2-CH_3$ | 1.4 | 0.06 |
| 16 | $-CH_2-CH_2-CH_3$ | 0.64 | 0.02 |
| 7 | $-(CH_2)n-$ | 0.40 | 0.03[c] |
| 2 | $-CH_2\,CO-$ | 0.26 | 0.02[c] |
| | $-(CH_3)_3-N-$ | 0.62 | 0.05 |

[a] From Godici, P. E. and Landsberger, F. R., *Biochemistry*, 13, 362, 1974.

[b] From Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., and Smith, I. C. P., *Biochemistry*, 15, 954, 1976.

[c] Calculated from $^{13}C$ data by method given in Saito, H., Mantsch, H. H., and Smith, I. C. P., *J. Am. Chem. Soc.*, 95, 8453, 1973.

interest because it was shown that the thermotropic phase transition is a gradual one with these lipids.[125] It was suggested that domains of crystalline chains grow within the bilayers as the temperature is lowered so that part of each chain is "fluid" and part is crystalline, but some comments on this interpretation have been made.[128] Various other lipid-water systems have also been examined.[129]

### 3. *Hydrocarbon Chain Organization*

We have already mentioned different hydrocarbon chain organizations that are found to exist in lamellar phases of phospholipids in water (see Figure 4). The detailed organization of the hydrocarbon chains of lipid mesophases both in the anhydrous and aqueous systems have been variously described as liquid-like and chaotic,[127] extended and ordered,[130] significantly ordered,[131] and partly ordered with some rotational isomerism.[132] The interpretation of the X-ray data associated with the short spacings is not unequivocal on this point.[128] The diffuse diffraction band at 0.46 nm from randomly oriented bilayers is similar to that obtained from liquid paraffins. This diffraction is too complex to be analyzed in terms of a detailed model for the organization of the hydrocarbon chains, and it is yet to be shown that it corresponds to a unique mode of packing of the chains. Warren[133] has discussed the origin of the diffraction band from liquid paraffins in terms of a cluster of seven hexagonally packed chains in all-*trans* conformation. This model has been substantiated, and values for the radial distribution of chains in the liquid have been derived.[134, 135] It was assumed, however, that liquid paraffin is an isotropic structure and this is clearly not valid in the case of lipid bilayers.

Calorimetric data has been used to examine the hydrocarbon chain motion above the phase transition temperature of anhydrous lecithins.[132] The heat involved in the transition is found to be about 95% of the total heat of fusion. The total entropy per methylene group is the same for all long-chain compounds in the crystalline form at their chain melting point. The entropy gain during the transition from $\beta$ crystal to isotropic liquid for *n*-alkanes, triglycerides, and fatty acids is 2.6 e.u. per $CH_2$ group, whereas for the crystal transition for lecithins the equivalent figure is 1.1 e.u. Thus, in the liquid crystalline state the chain fluidity is about half that found in liquid *n*-

alkanes at the transition temperatures. It was suggested that this may arise by inhibition of rotation about the carbon-carbon bonds due to the presence of the neighboring chains.[132]

Infra red[136,137] and Raman spectroscopic studies[138] both show that some rotational isomerism about the carbon-carbon bonds occurs in the anhydrous and lipid-water systems. Calorimetry of the fully hydrated lecithins reveal greater chain mobility above the transition temperature than do the anhydrous or monohydrate liquid crystals but still less than that which is observed in the *n*-alkane melt so that some order is retained and the chains are never in completely chaotic arrangements.[139]

Below the transition temperature the area per lipid molecule is about 0.48 $nm^2$ while above this temperature (at 40% by weighted water) the area per molecule is some 0.7 $nm^2$,[86,140] suggesting that there is still some order normal to the plane of the membrane. The theoretical studies of Whittington and Chapman[141] emphasized the steric repulsion of neighboring chains which limits $CH_2$ rotational freedom and makes such motion cooperative between these chains.

Electron spin resonance and deuterium magnetic resonance studies of phospholipids in aqueous dispersions have provided additional information about the conformation of the hydrocarbon chains. Both techniques can be used to determine angular fluctuations of individual chain segments, the so-called order parameter.[142] These fluctuations arise from rotational isomerizations about the carbon-carbon bonds which, because of the bond rotational potential, can exist in only one of three conformers, *trans*, *gauche*$^+$, or *gauche*$^-$. Hydrocarbon chains in all *trans* configurations are regarded as ordered whereas *gauche*$^\pm$ conformers introduce disorder into the chain. The average conformation of fully saturated hydrocarbon chains in bilayers is constrained by the energy difference between *trans* and *gauche* isomers (approximately 2 kJ $mol^{-1}$)[143] and is influenced by cooperative interactions between nearest neighbors as well as interactions with adjacent hydrocarbon chains. Additional constraints are imposed in the case of unsaturated hydrocarbon chains due to restricted rotation about *cis* double bonds. Seelig and Seelig[101] have pointed out that the fluidity of phospholipid bilayers is a function of the rate of motion of individual molecules, or at least the hydrocarbon chain component, and not the order in which these chains exist so that although the two parameters may be related the relationship is not necessarily a direct one. Spin-label analogues of phospholipids, in which the nitroso radical is attached to various positions along the hydrocarbon chain[144] and spin-label stearate probes,[145] have shown that all *trans* configurations predominate in the chain segments closest to the glycerol moiety and become progressively disordered towards the terminal region of the chain. Qualitatively similar results have been obtained from deuterium magnetic resonance studies of specifically deuterated phospholipids[101] and fatty acid probes[102,146] although these measurements provide considerably higher values of the order parameter than spin-label methods. This has been explained by the fact that bulky nitroxide-containing spin resonance probes perturb their environment whereas the deuterated lipids do not. Moreover, according to the deuterium studies the order of the hydrocarbon chains appears to be maintained further along the chain although both methods indicate equal disorder at the terminal region.[147-149] Studies of molecular motion and order in biological membranes using deuterium magnetic resonance spectroscopy have been reported.[100,150]

### *4. Phospholipid Monolayers at the Air-Water Interface*

A correlation has been established between the monolayer properties of lipids at the air-water interface and the properties of lipids in aqueous dispersions.[151,152] The condensed monolayer corresponds to the "gel-type" structure and the expanded monolayer to the "fluid condition" above the lipid transition temperature, so that similar

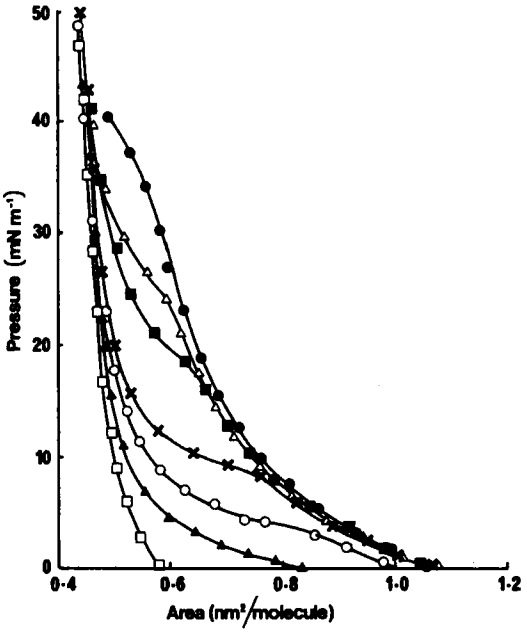



FIGURE 7. Pressure-area curves of 1,2-dipalmitoyl-phosphatidyl-choline. Phospholipid monolayers were spread on subphases of 100 mM NaCl maintained at various temperatures between 6.2°C and 34.6°C. Each curve represents the pressure-area relationship at a particular temperature; ●, 34.6°; ▲, 29.5°; ■, 26.0°; ×, 21.1°; ○, 16.8°; ▲, 12.4° □, 6.2°. (Data from Phillips, M. C., in *Progress in Surface and Membrance Science*, Vol. 5, Danielli, J. F., Rosenberg, M. D., and Cadenhead, D. A., Eds., Academic Press, New York, 1972, 139.)

thermotropic phase changes can be observed with the monolayers.[153] All monolayer states are possible with the saturated lecithin and phosphatidylethanolamine homologues.[152] Thus if the hydrocarbon chains are sufficiently long, condensed monolayers are formed whereas liquid-expanded films are formed with shorter chains. These two limiting states are sufficiently well defined so that at any particular temperature only one of the homologues studied exhibits the transition state. This has been well characterized in the case of 1,2-dipalmitoylphosphatidylcholine which appears to exist in two forms at 22°C depending on the surface pressure of the monolayer.[140] Thus at pressures less than 10 mN $m^{-1}$ typical expanded type monolayers are observed whereas above this pressure there is a transition to a more condensed form. The pressure at which this transition is observed is related to the temperature of the system as illustrated in Figure 7, which shows how temperature changes can give rise to the above physical states in a monolayer of a single homologue. At the lowest temperature observed, 6°C, the film is condensed at all pressures and at 35°C it is expanded at least up to a pressure of 30 mN $m^{-1}$. It is likely that at the phase transition temperature of the phospholipid (41°C) the film would remain in an expanded state even at the collapse pressure of the monolayer.

The molecules in a completely condensed phosphatidylethanolamine film are much more closely packed than are those in the equivalent lecithin monolayer. This can also be correlated with the bulk properties. The lecithins have a lower transition temperature than does the equivalent chain-length phosphatidylethanolamine. This presumably

arises from steric factors associated with the large polar groups on the lecithin molecules. Marcelja[149] has used this correlation between the monolayers and lipid-water dispersions to calculate the lateral pressure in the hydrocarbon chain region, and this he estimates to be of the order of 20 mN $m^{-1}$ for each half of the bilayer. Lipid phase transitions have been studied in planar lipid bilayer systems after creating domains of fluid and domains of rigid lipid.[154]

## B. Molecular Motion of Lipids in Bilayers

### *1. Mobility in the Lamellar Plane*

Physical studies of phospholipid dispersions have shown that the lipid molecules in fluid bilayer membranes readily diffuse in a lateral direction in the plane of the bilayer,[155] each molecule exchanging with its neighbor about $10^6$ times per second.[156] Similar studies of lateral diffusion rates have been applied to biological membranes. Lateral diffusion rates of a spin-labeled phosphatidylcholine analogue incorporated into sarcoplasmic reticulum membranes and dispersions of lipids extracted from these membranes have been determined.[157] The spin-label method relies on the fact that electron spin resonance lines become broadened in a concentration-dependent manner due to spin-spin interactions between paramagnetic probe molecules. It has been suggested that spin-exchange interactions resulting from collisions between probe molecules are the predominant cause of resonance line broadening at temperatures of more than about 40°C. However, the interpretation of signal line shapes is somewhat complicated because at the high spin-label concentrations conventionally employed, both dipole-dipole and electron exchange interactions contribute to line broadening. Nevertheless, the collision frequency between spin-label molecules has been calculated from spectral line shapes at various probe concentrations assuming an average area for each lipid molecule in the bilayer of 0.6 $nm^2$. The lateral diffusion of spin-label molecules incorporated into sarcoplasmic reticulum membranes was $0.6 \times 10^{-11} m^2 sec^{-1}$, a value almost identical to that obtained for the probe interpolated into aqueous dispersions of a total membrane lipid extract ($10^{-11} m^2 sec^{-1}$). The latter measurement compared almost exactly with diffusion rates of spin-label probes in artificial phosphatidylcholineolesterol bilayer dispersions.[156] It is apparent from these measurements that phospholipids diffuse in the plane of some biological membranes as rapidly as they do in artificial bilayer membranes. It should be emphasized that mobility measurements only refer to the lipid domain accessible to the probe molecules, and significant errors could arise if probe molecules were restricted to, say a fluid as distinct from a rigid region of the membrane.

### *2. Phase Separations*

The use of spin labels such as TEMPO to establish a phase separation of mixed lipid systems has been reported by Shimshick and McConnell.[110] Other phase separation properties have been observed by Ito and Ohnishi[158] who showed that lipid phase separation in phosphatidic acid-lecithin membranes can be induced by $Ca^{2+}$. The tendency of stearic acid spin-label probes to migrate to the more fluid lipid phase in multiphase systems has also been reported.[159] This confirms the earlier conclusions that measurements of membrane fluidity in heterogeneous systems are not necessarily representative of the entire membrane.[160]

Binary mixtures of lecithins such as distearoyl and dipalmitoyl lecithin (DSL-DPL) and also distearoyl lecithin and dimyristoyl lecithin (DSL-DML) have been examined by calorimetry. With the DSL-DPL mixtures the phase diagram shows that a continuous series of solid solutions are formed below the $T_c$ line suggesting that this pair of molecules co-crystallize, probably due to the small difference in chain length between the lipid molecules. With the system DSL-DML, monotectic behavior was observed

with limited solid solution formation. Here the difference in chain length is already too great for the lipids to cocrystallize so that as the system is cooled lecithin molecules migrate within the bilayer to give crystalline regions corresponding to the two compounds.[162]

Examination of a series of fully saturated lecithins with dioleoyl lecithin gave similar results with phase separation of the individual components taking place.[161] Calorimetric studies of mixed lecithin-cerebroside systems and on lecithin phosphatidylethanolamine mixtures[162-164] have also been reported. The lecithin-phosphatidylethanolamine systems of the same chain length give a wide melting range with some separation of the different lipid classes.

*3. Transbilayer Motion of Lipids*

All the available evidence suggests that, in contrast to the freedom of lipid molecules to move laterally in the plane of fluid bilayer structures, the movement of molecules from one leaflet of the bilayer to the other is a comparatively rare event. The reason for this constraint lies in the physical properties of the constituent molecules. All lipid molecules are amphipathic, and the lowest free energy of the bilayer structure is achieved when the polar regions of the molecules are in a polar environment. In order to migrate from one leaflet to the other, the polar group must traverse the hydrophobic interior of the bilayer while at the same time exposing some of the hydrocarbon residues to water.

The first indication that transbilayer migration is a relatively slow process was derived from studies of stearic acid films[165] and then phospholipids in model membrane systems.[166,167] Kornberg and McConnell[166] prepared dispersions of phospholipids containing a spin-label group attached to the choline residue of lecithin. When the impermeant reducing agent, ascorbic acid, was added to a suspension of vesicles, the signal arising from molecules residing on the outer leaflet of the bilayers was abolished, and the decay of the residual signal provided a measure of the rate at which spin-label phospholipid moved from the inner to the outer leaflet of the bilayer vesicle. When the same technique was applied to biological membranes,[166,168] the transmigration rate of the spin-label analogue was considerably faster than that observed with vesicles of pure phosphatidylcholine suggesting that the assembly of different components, both lipid and protein, may influence the rate of transmigration of individual components. The rate of transmigration observed in these experiments is of the spin-label phospholipid analogue, and it is likely that the corresponding rates for phospholipid molecules may be somewhat different because the spin-label itself modifies the physical properties of the molecule.

Evidence for a slow transmigration of phospholipids has also been obtained from nuclear magnetic resonance spectroscopic studies.[169,170] When paramagnetic ions such as $Mu^{2+}$, $Eu,^{3+}$ or $Pr^{3+}$ are added to bilayer dispersions of phospholipids or mixtures of phospholipids and cholesterol, the phosphorus or proton resonances are modified in a characteristic manner depending on the particular ion, the environment in which they are located, and the nature of the chemical groups with which they interact. Since these ions are relatively impermeable to phospholipid bilayers when present in low concentration, perturbation of the resonance signal is restricted to interacting groups located in the outer leaflet of bilayer. As with the spin-label experiments, there is an immediate effect on those signals arising from phospholipids exposed to the perturbing ions on the outside of the vesicles while the remaining signals are selectively perturbed at a slow rate related directly to the rate of migration of phospholipid molecules from the inner to the outer leaflet of the bilayer.

A somewhat different approach has been to determine the rate of exchange of radioactively labeled lipid components between biological membranes and lipid dispersions.

Pozansky and Lange[171] measured the rate of exchange of radioactively labeled cholesterol between erythrocyte ghosts and sonicated dispersions of mixtures of phosphatidylcholine and cholesterol. They found that only 71% of the cholesterol pool present in the vesicles was readily exchanged, and it was suggested that this represented those molecules present in the outer leaflet of the membrane bilayers. When cholesterol is transferred from erythrocytes to liposomes, these molecules remain in the readily exchangeable pool and presumably do not migrate readily to the inner leaflet of the bilayer. Quantitative estimates of the intermixing of the two cholesterol pools in the bilayer suggested that migration from one leaflet to the other had a half time of at least 6 days. Similar types of experiments have been described to measure rates of phosphatidylcholine transposition across mitochondrial membranes[172,173] except that purified phospholipid exchange proteins were used to facilitate phospholipid transfer between phospholipid dispersions and mitochondria. When liposomes prepared with $^{32}$P-labeled phosphatidylcholine were incubated with excess mitochondria and purified exchange protein, only about 60% of the phosphatidylcholine was rapidly transferred to the mitochondria. The remaining 40% was exchanged at a much slower rate (half-time of several days) and was presumed to represent the transposition of phospholipid molecules from the inner leaflet of the bilayer to the rapidly exchanging pool located in the outer leaflet.

## C. Lipid Interactions

### *1. Phospholipid and Cholesterol Interactions*

A number of physical studies have demonstrated that the hydrocarbon chains of lecithins in water have less thermal motion in the presence of cholesterol than they have in the absence of cholesterol above the $T_c$ line, and they have more thermal motion than in the absence of cholesterol below the $T_c$ line. X-ray evidence indicates a lamellar arrangement with up to about 50% cholesterol whereupon an additional long spacing pattern is observed due to the separation of crystalline cholesterol.[174] These results may be interpreted in terms of penetration of the lipid bilayer by cholesterol. As we have noted above, the hydrocarbon chains of lecithin lamellae in water are hexagonally packed and tilted at 58°C. It can be envisaged that penetration will be facilitated when the chains are vertical; this causes an increase in the X-ray long spacing. At concentrations of cholesterol greater than 7.5% the long spacing then decreases. Above this critical concentration there is a reduction in the cohesive forces between the chains producing chain fluidization. Proton magnetic resonance studies of egg-yolk lecithin shows that the addition of cholesterol produces effects which depend upon the physical state of the lipid.[175] At temperatures greater than the transition temperature, the addition of cholesterol reduces the signal intensity and hence the motion of the methylene protons of the hydrocarbon chains. The addition of cholesterol to sonicated lecithins also appears to broaden the choline residue signal to a lesser extent than it does that of the alkyl chains suggesting a more pronounced effect on hydrocarbon chain immobilization.[176] Linewidth measurements clearly demonstrate that the fluidity of the chains in the presence of cholesterol is intermediate between that of aqueous lecithin below and above its transition temperature. Even in aqueous lecithin below its transition temperature, the choline group still retains appreciable mobility.

Unsonicated dispersions of dipalmitoyl lecithin and cholesterol have been studied by wide-line NMR spectroscopy. The observed linewidths provide a good indication of the mobility of the system. Analysis of the relative intensities of the observed signals allows the location of the steroid within the bilayer to be postulated as between the hydrocarbon chains with the steroid hydroxyl group adjacent to the phosphate group of the lecithins.[176]

Phase diagrams for the system dimyristoyl lecithin-cholesterol-water constructed from spin-label studies[177] show evidence of solid phase immiscibility but no indication of complex formation. Because of the inherent solid phase immiscibility, it was possible to explain the loss of the transition when 50% cholesterol is present. When the lipid is above its transition temperature and cholesterol is added, the system passes from fluid to mixed fluid and solid, to all solid as the steroid concentration increses. There have been many other studies of cholesterol interactions with lipid systems using the spin-label method (see, for example, Reference 178).

The phase transitions of sonicated vesicles of dipalmitoylphosphatidylglycerol and dipalmitoyl lecithin in the presence and absence of cholesterol have been studied by fluorescence polarization and permeativity of $^{22}Na^{+}$.[179] Polarization results indicate that a perylene probe is able to penetrate the membrane interior only at temperatures in excess of the transition temperature of the lipid, and at this temperature a local maximum occurs in the diffusion rate of $^{22}Na^{+}$. The absence of such changes on addition of cholesterol indicates that this removes the gross phase transition.[180] The effect of cholesterol on phase transition behavior of mixed dipalmitoyl lecithin-cholesterol bilayers has been investigated.[181] In bilayers containing less than equimolar amounts of cholesterol, discrete regions of 1:1 complex separate out leaving lecithin molecules at the boundary of these regions for which cooperative motions are not possible and clusters of free lecithin molecules which freeze at the normal transition temperature. Discussion of the molecular associations that exist between hydrocarbon chains and cholesterol is deferred to a later section.

### *2. Interaction of Lipids with Metal Ions*

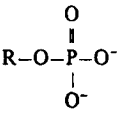
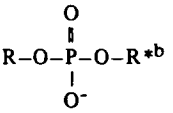
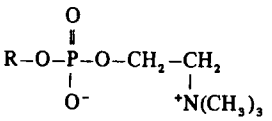
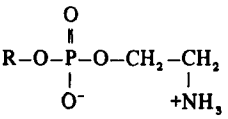
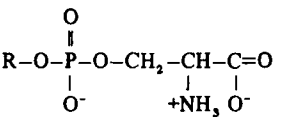
The stability of lipid bilayers depends on both the segregation of hydrocarbon residues from the aqueous phase and on a polar interaction of the lipid with water. Polar interactions usually involve a charged group on the lipid but in some lipids, neutral sugars dominate the hydrophilic region of the molecule. Phospholipids, for example, bear a partial negative charge on the phosphate group that usually exhibits a pKa of about 2 or 3. The structure and electrostatic properties of phospholipids are presented in Table 14. Apart from phosphate groups, other charged groups include sulfate (cerebroside sulfate), carboxyl (phosphatidylserine), amino bases (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin), and amino sugars (gangliosides). The polar region of cholesterol arises from a single hydroxyl group which serves to orient the molecule in lipid bilayers.

When acidic phospholipids are oriented as interfacial monomolecular films or in bilayers, the charged groups reside in a plane near the junction of the aqueous and hydrocarbon residues. The negative potential generated by dissociation of the phosphate groups depends on the charge density and degree of dissociation and decreases exponentially with distance from the plane of the ionizable residues. The presence of similarly charged groups on adjacent molecules leads to a considerable increase in cross-sectional area occupied by each molecule because of electrostatic repulsion. Moreover, in bilayers the electrostatic repulsion between similarly charged planes on each surface prevents thinning of the structure and supplements the lateral cohesive forces between hydrocarbon chains, thus stabilizing the two layers.

The charged groups of the lipid attract counter ions, sometimes referred to as Gegen ions, from the aqueous phase, and in the absence of specific interactions these appear to be arranged according to a Boltzmann distribution. Certain ions, such as $UO_2^{2+}$, interact more specifically with the ionized groups of lipids so that in addition to coulombic forces of attraction, van der Waals and other dipole-dipole forces promote a more intimate association between the charged components. In the case of zwitterionic

Table 14

**The Structure of Phospholipid-Charged Groups and Their Electrostatic Properties**

| Phospholipid ionizable groups | Structure[a] | Electrostatic properties |
|---|---|---|
| Primary phosphate (phosphatidic acid) |  | $pK_1$ 3.9<br>$pK_2$ 8.3 |
| Secondary phosphate (phosphatidylinositol cardiolipin) |  | pK < 2.0 |
| Seconary phosphate + quaternary amine (sphingomyelin phosphatidylcholine) |  | Isoelectric in pH range 3 to 10 |
| Secondary phosphate + primary amine (phosphatidylethanolamine) |  | Net negative at pH 7.4 |
| Secondary phosphate + amine + carboxyl (phosphatidylserine) |  | Net negative at pH 7.4 |

[a] R = 1,2-diacylglyceride.
[b] R* = *myo*-inositol or phosphatidylglycerol.

phospholipids like lecithins and sphingomyelin, the presence of a secondary phosphate group and a quaternary amine leads to an internal salt linkage, and coulombic attraction of counterions does not appear to take place. Nevertheless, there is evidence to indicate an interaction of lecithins with uranyl ions ($UO_2^{2+}$)[182] brought about by forces other than simple charge-charge attraction. Another factor which influences the distribution of counterions is the nature of the binding of ions to the charged lipids. Divalent ions such as $Ca^{2+}$ apparently occupy a binding site consisting of two adjacent lipid molecules separated by a distance equivalent to the diameter of the hydrated ion.[183] The surface potential thus conforms to a Stern type arrangement with $Ca^{2+}$ counterions rather than a Boltzmann distribution predicted by the Gouy relationship. Metal ion interactions have been known for some years to affect the thermotropic phase transition of soap systems. The thermotropic phase transition of stearic acid takes place at 114°C with the sodium salt and at 170°C with the potassium salt. These phase transitions can be linked to the monolayer characteristics.

Early studies of stearic acid monolayers showed that interaction with $Ca^{2+}$ ions caused an increase in surface pressure (i.e., condensation) and also decreased the permeability to water. The same effect has been observed with phosphatidylserine monolayers,[184] but $Na^+$ and $K^+$ addition gave no such condensation. Later, more extensive studies showed that a variety of acidic phospholipid monolayers undergo an increase in surface potential and decrease in surface pressure on addition of $Ca^{2+}$ and other bivalent cations.[185] Phosphatidylserine appears to be more selective than phosphatidic acid, but for both systems the order of cation effectiveness is

$$Ca^{2+} > Ba^{2+} > Mg^{2+}$$

The formation of linear polymeric complexes was proposed to account for these findings.

Cationic charge has been observed to be important in some bilayer studies of phosphatidylserine.[186] Black membranes formed in the presence of $Ca^{2+}$ ions are found to be more stable with a higher electrical resistance than those formed in the presence of $Na^{+}$ only. The concentrations of cationic species required to produce charge reversal in phosphatidylserine dispersions have been determined together with association constants for the species formed.[187] The results obtained agree well with those obtained previously. Uranyl cation is exceptional in that this ion causes an increase in the thermotropic phase transition temperature of lecithins.[188] Thus two main phase transitions are observed corresponding to the presence of complexed and uncomplexed lipid, but when the titration is complete only the higher melting transition remains. Interaction between cations and phosphatidylserine causes even greater shifts of transition temperature than is observed with lecithin molecules, and all the cations studied were found to increase the phase transition temperature of phospholipids.[188] A number of studies have also shown that the type of anion[164] as well as cations[189-194] and pH[195] can affect the thermotropic phase transition temperature of various purified phospholipids. Trauble et al.[196] have studied the effects of pH and univalent cations on lipid bilayer structures. They show that electrostatic interactions at charged lipid bilayers make a significant contribution to the free energy of the system. They show that with some lipid systems the main phase transition of the lipid can be shifted at constant temperature by variations of pH and salt concentration. An adequate quantitative description of this behavior is obtained from the Gouy-Chapman theory. They point out that by varying molar electrostatic free energy, $G^{el}$, of a membrane fluidization or condensation can be produced; and, furthermore, for a lipid bilayer system which is initially mixed (or separated into phases), a decrease (or increase) in $G^{el}$ can produce phase separation (or mixing).

*3. Drug Interactions*

The precise biological and pharmacological actions of drug molecules are largely undetermined. There is, however, widening belief that interaction with the lipid constituents of membranes resulting in an alteration of the fluidity characteristics may be an important mode of action of the drugs. It has been shown, for example, that drug molecules can affect the thermotropic phase transition of lipid-water systems. Sometimes this corresponds to a removal of the transition somewhat similar to that observed with cholesterol. As examples, we can cite gramicidin A[197] and other antibiotic molecules.[198] On the other hand, sometimes the drug shifts (with increasing concentration) the lipid transition temperature to lower values, and we can instance a range of antidepressant drug molecules such as desipramine.[199] Hill[200] has studied the effects of a series of normal alcohols (up to $C_8$) as well as three inhalation anesthetics on the lipid phase transition temperatures and observed shifts both to lower and higher values. Electron spin resonance studies of the effects of inhalation anesthetics on the order parameter of nitroxide-substituted egg-yolk lecithin bilayers indicates a more disordered bilayer, the degree of disorder being linearly dependent on dose within the concentration range employed for clinical anesthesia.[201] Furthermore, high pressures of helium appear to antagonize the effect of the anesthetic paralleling the known pressure reversal effect observed with these substances.[202]

Certain other drugs when added to membranes bind to particular lipids, and the resulting complex effectively removes the lipid from any specific interactions with membrane proteins. The polyene antibiotic filipin, for example, forms a complex with

cholesterol when added to membranes. Phospholipids, on the other hand, form a complex with melittin, a component of bee venom, which is a peptide of 26 amino acid residues of which 20 are hydrophobic or neutral in character. The effect of these drugs on adenylcyclase activity of avian erythrocytes has been investigated by Puchwein et al.[203] They found that when filipin was added to these membranes in amounts that did not disrupt the membrane structure catecholamine activation of adenylcyclase could be completely abolished even though hormone binding to the receptor site was apparently unaffected. The observations were consistent with an uncoupling of the hormone-receptor complex from the catalytic site because activation of the enzyme by fluoride was unaffected by the drug, and hormone activation could be fully restored on removing filipin thereby demonstrating that the receptor site was not damaged by the drug. The effect of melittin on catecholamine activation of avian eyrthyrocyte adenylcyclase was also investigated. When melittin was added to the membrane in a ratio of 1:40 molecules of membrane phospholipid, there was almost complete loss of hormone activation of the enzyme; fluoride stimulation was slightly enhanced under these conditions. It was concluded from these experiments that membrane lipids were required to maintain the hormone receptor and the catalytic site of adenylcyclase in an active configuration.

### D. Summary and Critique

Studies of the properties of lipid extracts of biological membranes and synthetic lipids and cholesterol in aqueous systems provide a useful and informative insight into the behavior of these constitutents in membranes. Studies of these systems have, for example, led to the concepts of fluidity and of phase transitions, diffusion of lipids in bilayer structures, and phase separation behavior. Physical studies have also revealed precise detail of the motion of lipid molecules arranged in bilayer configuration, including estimates of the rate of transbilayer motion of the lipid molecules. The polar interactions of charged lipids with metal ions and $H_3O^+$ has been the subject of many investigations, and information concerning these interactions and phase transition and phase separation has been reported. Information about the water structure close to the boundaries of the lipid structures has also been obtained.

The degree to which the behavior of the model systems mimic the properties of cell membranes is gratifying. Even with complicated systems containing other molecules such as cholesterol or protein, the behavior is often paralleled by the natural biomembranes. An example of this is the lipid phase separation from intrinsic protein components which tend to aggregate as the membrane is cooled to temperatures where the lipid crystallizes. In the presence of appreciable amounts of cholesterol in the membrane, however, protein aggregation is prevented. Many other questions regarding the relationship between model membrane systems and biological membranes remain. A number of these relate to effects that can be readily detected in well-defined lipid systems, but their existence and/or importance in biomembrane function can only be surmised at this time. For example, do effects on the physical properties of bilayer lipids triggered by changes in pH or the interaction with metal ions play a role in biomembrane function such as signal transduction?

## IV. INTERACTIONS OF THE MEMBRANE COMPONENTS

If we consider cell membranes as consisting of a lipid bilayer matrix into which the other membrane components (cholesterol and proteins) are intercalated, important questions are raised concerning the nature of the interactions which exist between these components. The study of model membranes either in the form of simple lipid-water dispersions or reconstituted systems has proved of value for this purpose. In this sec-

tion we review the type of information that can be obtained concerning the various types of interaction between the membrane components by using model and reconstituted systems and then examining the effect of certain drugs and enzymes on modifying these interactions.

## A. Lipid-Water Systems Containing Another Component

### *1. Cholesterol-Lipid Interactions*

Cholesterol is a constituent found in varying amounts in a number of cell membranes, and consequently many studies of this molecule have been undertaken to define its role in membrane structures. The molecule is relatively insoluble in water, but it can be dispersed in a lipid structure and transported by the lipid in an aqueous environment. Studies of model phospholipid bilayer structures show that the cholesterol molecules in these systems are interpolated among the lipid chains of the bilayer.

Studies of mixed monolayers of lipid and cholesterol showed more than 50 years ago that the effect of cholesterol on the lipid was to cause a reduction in the mean molecular area of the two components, creating thereby a "condensed" system. This condensing effect was explained in different ways, some associating it with a specific position of the double bond (the $\omega 9$ position) or a *cis* double bond in the lipid chains, or both, while others suggested that the presence of the cholesterol caused an increase and some a decrease in lipid viscosity.

The controversy was eventually resolved when the effect was considered by Chapman and co-workers in terms of the influence of cholesterol on the lipid fluidity and in particular how cholesterol affects the rotational isomerism of the $CH_2$-groups of the lipid chains. The action of cholesterol was also shown to be related to the physical state of a pure phospholipid monolayer. Generally an expanded monolayer is condensed by the addition of cholesterol, and this is associated with an effect upon the hydrocarbon chain mobility. This is greatest when the phospholipid is close to the transition temperature for the change from condensed to expanded monolayer. The position of *cis* double bonds is not a necessary condition for this condensation effect, and even the presence of a double bond is not required for this effect. This is illustrated in Figure 8.[204,205] The interpretation of the monolayer properties in terms of an effect of the cholesterol molecule on lipid fluidity has been confirmed and extended to lipid bilayer structures using other physical techniques such as NMR and ESR spectroscopy as well as fluorescence methods.[206,207]

Studies of cholesterol-lipid interactions at temperatures where the lipid would be below its transition temperature have been made using a variety of techniques. X-ray and calorimetric studies[208] showed that the addition of cholesterol to dipalmitoyl lecithin decreases the transition temperature and reduces the heat absorbed at the transition. No transition is observed with equimolar ratios of lecithin and cholesterol capable of incorporation into the system before cholesterol precipitates. The calorimetric curves and the other data, i.e., transition temperature, heat absorbed in the lipid transition, heat absorbed at 0°C, and the X-ray long spacings at 25°C, are shown in Figure 9. Also shown is a schematic representation of the structures in the presence of varying amounts of cholesterol.

Other workers have confirmed this effect of cholesterol upon the lipid phase transition, and it has been suggested that the point at which no heat is absorbed may correspond to a value of 33 mol of cholesterol per 100 mol phospholipid. The various studies of cholesterol in the lipid lamellar-water systems have led to the conclusion that cholesterol keeps the lipids in an "intermediate fluid" condition. Thus, at a temperature where the lipid would normally be above its transition temperature ($T_c$), the presence of cholesterol appears to cause a reduction of lipid fluidity while at a temperature below $T_c$ the presence of cholesterol tends to increase the lipid fluidity. The presence

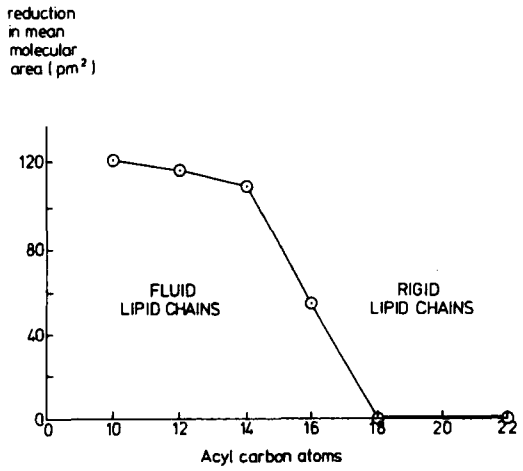



FIGURE 8. Condensing effect of cholesterol on monolayers of a homologous series of saturated 1,2-diacyl-L-phosphatidylcholines. The reduction in mean molecular area from that of an ideally mixed system of equimolar amounts of phospholipid and cholesterol is plotted as a function of hydrocarbon chain length. (Data from References 204 and 205.)

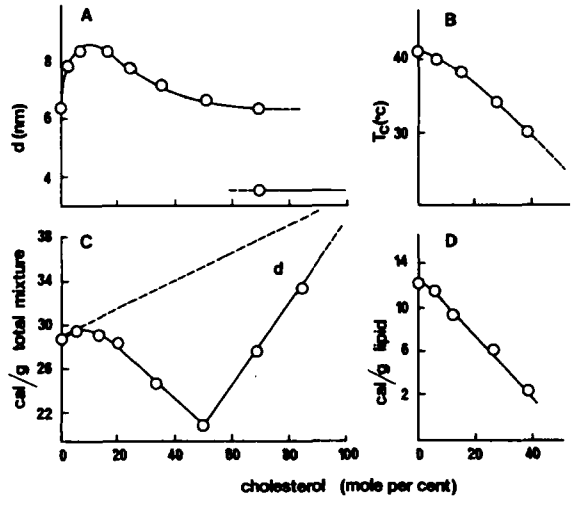



FIGURE 9. The variation with 1,2-dipalmitoyl-L-phosphatidylcholine-cholesterol ratio in 50 wt 2% aqueous dispersions of (A). X-ray long spacing (d) at 25°C (B). Transition temperature ($T_t$), (C). Heat absorbed in the ice transition at 0°C (D). Heat absorbed in the phospholipid transition. (Data from Ladbrooke, B. D., Williams, R. M., and Chapman, D., *Biochim. Biophys. Acta*, 150, 333, 1968.)

of cholesterol, therefore, is to modulate the fluidity of the lipid hydrocarbon chains, but the polar interaction of the lipid with water may also be affected.[209]

The question of how cholesterol is arranged in the plane of phospholipid bilayers is not resolved. The existence of stoichiometric complexes of phospholipid and cholesterol in ratios of 1:2,[210] 1:1,[211-213] and 2:1[214,215] have been proposed; e.g., when there is more lipid than 2:1 complex, the system is generally regarded as consisting of a

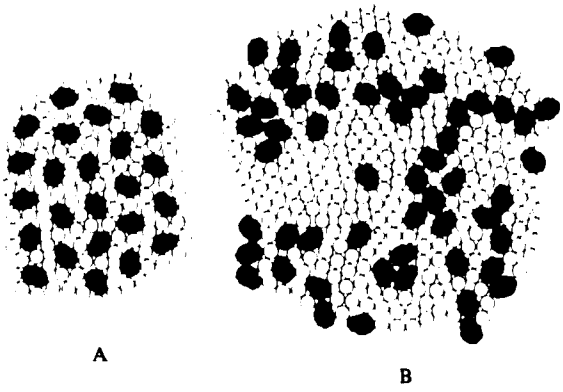



FIGURE 10. (A) An arrangement of lipid and cholesterol molecules corresponding to a 2:1 phospholipid cholesterol complex as suggested by Engleman and Rothman.[214] (B) An experimentally simulated random array of lipid and cholesterol in the same ratio as in A.

complex coexisting with the excess lipid. The consequences of random arrangements of lipid and cholesterol have recently been explored.[216] A comparison between a 2:1 complex of lipid and cholesterol after Engleman and Rothman, and a random arrangement of the components in the same ratio, are presented in Figure 10. A feature of the random array is the existence of cholesterol-cholesterol contacts even at relatively low amounts of cholesterol. (A cholesterol on average contacts one other cholesterol at 25 mol cholesterol per 100 mol lipid.) With the 2:1 lipid-cholesterol complex model, such cholesterol contacts can only take place when the proportion of cholesterol in the mixture exceeds 33 mol per 100 mol phospholipid presumably when there is insufficient lipid to completely form only these complexes.

Nuclear magnetic resonance studies using $^{13}C$ labeled cholesterol in lipid dispersions shows the presence of two components at lecithin-cholesterol ratios of less than 4:1.[217] If this result is correct, it is irreconcilable with cholesterol forming either a 1:1 or 1:2 complex with lecithin. Unfortunately, this datum seems to contradict other nuclear magnetic resonance data using deuterated cholesterol which shows only one signal at 1:1 molar ratio of cholesterol to lipid.[218]

### *2. Polypeptide-Lipid Interactions*

There are sometimes advantages in using model systems to examine protein-lipid interactions in well-characterized polypeptides incorporated into lipid structures. This has led to experiments designed to mimic electrostatic interactions and hydrophobic interactions. The fact that certain proteins are considered to exist in some membranes in trans bilayer helical polypeptide segments also encourages belief in the relevance of such studies.

Electrostatic interactions of lipids and polypeptides have been studied by various workers. Chapman et al.[219] have reported the binding of polylysine to phosphatidylserine and Trauble and Eibl[220] examined the effect of interacting polylysine with phosphatidic acid. Effects upon the lipid transition temperature $T_c$ were observed. Bach and Miller[221] also studied the interaction of basic polypeptides (copolymers of lysine with phenyl/alinine or tyrosine with phosphatidylserine) using calorimetry. More recent studies of Bach et al.[222] on the circular dichroism of the random copolymers of L-lysine with L-phenyl alanine, L-tyrosine, and L-serine; i.e., $(Lys,Phe)_n$, $(Lys,Tyr)_n$, and $(Lys,Ser)_n$ showed that they undergo conformational changes on interaction with phos-

pholipid vesicles. For example, $(Lys,Phe)_n$ appears to undergo a conformational transition from random coil to $\beta$-structure whereas the copolymer $(Lys,Ser)_n$, on interaction with either aqueous dispersions of phosphatidylserine or phosphatidylcholine, and $(Lys,Phe)_n$, interacting with vesicles of phosphatidylcholine, remain in the random coil configuration.

The interaction of synthetic polypeptides with lipids has been investigated using black lipid films,[223,224] monolayers,[225] and phospholipid vesicles.[226,227] The electrical resistance of black lipid membranes, for example, is substantially reduced and the capacitance altered by hydrophobic basic polypeptides, while the same bilayers are only slightly affected by the more hydrophilic basic polypeptides.[223] Invariably the effects are observed only after a relatively long exposure of the bilayer to the polypeptide solution. Hammes and Schullery[226] studied the interaction of vesicles of phosphatidylserine with various polypeptides. They observed that a complex was formed between phosphatidylserine and poly (L-lysine) and also between phosphatidylserine and poly (L-ornithine); polypeptides bearing negative charges, for example, poly (L-glutamic acid), did not appear to interact with phosphatidylserine or lecithin over a wide pH range. Yu et al.[227] have studied the interaction between poly (L-glutamic acid) and phosphatidylcholine vesicles.

Hydrophobic interactions of lipids and polypeptides have also been studied using polypeptides such as alamethicin and gramicidin A. Some of these polypeptides, e.g., gramicidin A and alamethicin, are known to form channels bridging the width of the bilayer.[228-230] In agreement with this idea is the finding that freezing the lipid below its transition temperature does not markedly affect the ability of gramicidin A to facilitate ion transport across lipid bilayers.[231] The intrinsic polypeptide, gramicidin A, when incorporated into a lipid bilayer, for example, smears out the lipid phase transition in a manner somewhat similar to that caused by cholesterol.[232] In a mixed lipid system the high melting lipid crystallizes first and causes an aggregation of the polypeptides. The presence of the polypeptide in the fluid state of the lipid (above the transition temperature, $T_c$) inhibits some of the chain movements of the lipids. The high surface activity of alamethicin is indicated by its propensity to orient at the lipid-water interface[230] and from microelectrophoretic mobility of phosphatidylcholine dispersions in the presence of the drug which acquire a net negative charge.[233] Moreover, alamethicin produces voltage-independent conductance changes in black lipid membranes consistent with the formation of pores in the membrane and reduces the motion of the lipid alkyl chains.[230] Preliminary observations on the ability of alamethicin and other channel-forming antibiotics to interact hydrophobically with lipid alkyl chains using differential scanning calorimetry have been confirmed more recently.[234]

The antibiotics nonactin and valinomycin also conduct ions across lipid bilayers, but rather than forming pores across the membrane they act as mobile ion carriers diffusing freely through the bilayer. A detailed study of the effects of valinomycin on lecithin bilayers using Fourier transform and pulsed nuclear magnetic resonance spectroscopy has shown that 0.2 mol% of the ionophore lowers the phase transition temperature of dipalmitoyl lecithin by approximately 1°C; the transition also takes place over a wider temperature range than in the absence of the drug.[235]

The hydrophobic peptide of glycophorin from human erythrocyte membrane, which penetrates bilayers of phospholipid, has also been found to increase the permeability of liposomes to $K^+$ and black lipid membranes to water and ions.[236] In conjunction with freeze-fracture electron microscopic evidence, it was suggested that the peptide produces a local disordering of the hydrocarbon chains of the bilayer phospholipids.

### *3. Protein-Lipid Interactions*

The binding forces and nature of interaction between lipids and various membrane-

associated proteins has been investigated in model systems, in reconstituted membrane systems, and in intact membranes, and we shall consider each of these systems separately. Each technique that has been employed in these studies has its own limitations and particular advantages; however, by collating information derived from a number of different methods, an overall view of the conditions governing these interactions can be obtained.

Let us first consider the likely interaction forces and then some of the experimental data that provide information on these forces.

A detailed discussion of the importance of the various different binding forces in protein-lipid interactions has appeared elsewhere.[237] Several general observations can be made however, concerning these interactions. Firstly, because individual lipid and protein molecules can be extracted from biological membranes with apolar solvents and detergents, respectively, the existence of covalent bonds between phospholipids and proteins are at most infrequent. Electrostatic interaction between lipids and proteins has been postulated in the model of the plasma membrane[238] and has been observed in certain model complexes. Polarization interactions are expected to be very weak. Dispersion interactions are usually small, and the effects diminish markedly with distance. Nevertheless, with lipid chains close-packed in a parallel configuration, these dispersion interactions are additive and can be as much as 50 to 100 kJ $mol^{-1}$, giving rise to important physical properties such as mesomorphic phase transitions.

Evidence for the importance of hydrophobic interactions has come from the theoretical work of Scheraga and his colleagues[239,240] as well as from experimental approaches emphasizing the effects of nonaqueous solvents,[241] hydrocarbons,[242,243] and fatty acids[244] on proteins and the interactions of nonpolar polypeptides.[245] X-ray studies of the water soluble proteins have shown that the folding of the polypeptide chain gives rise to a clathrate-type structure whereby water is excluded from the interior, where the major portion of the hydrophobic amino acids reside. Hydrophobic interactions are also important in the stability of the lipid bilayers in water since water is excluded from the central region of structure occupied by the hydrocarbon chains when the lipids orient in a bilayer configuration. The polar groups in this arrangement are afforded maximum exposure to the water. It has been suggested by Kauzmann[246] that the stability of such structures can be attributed to entropic effects since the exposure of hydrocarbon groups to an aqueous environment requires an increase in the order or quasi-crystalline order of water molecules about these groups. The removal of nonpolar groups from water results in a considerable increase in free energy due to an increase in entropy as water molecules in the system are permitted to assume more disordered configurations. It has been estimated that a change in free energy of about 10 to 20 kJ per nonpolar amino acid side chain or 4 kJ per $-CH_2-$ group results from the transfer of the respective groups from water to a nonpolar environment.

Another interaction which has been postulated to be important in protein-lipid interactions, and must also be considered, is that of hydrogen bonding. In the presence of water the hydrogen bond donor and acceptor groups (such as C=O and N–H groups of the peptide bonds) are often subject to competition for hydrogen bonding by the water molecules. In a nonaqueous environment, hydrogen bonding of such groups is, however, more favored.

The types of interaction between proteins and lipids oriented as monomolecular films at air-water or oil-water interfaces have been studied in some detail.[247] The advantage of such systems is that the lipid molecules are oriented and arranged in a well-understood manner, and their density at the interface can be readily altered by careful expansion or compression of the film. A number of surface parameters can be used to ascertain the extent and nature of the interaction with proteins introduced into the bulk phase. Thus, an increase in the surface pressure of a film upon adding protein is

generally assumed to represent the penetration of part at least of the space-occupying "volume" of the protein into the film, although this may not reach to the hydrophobic fatty acid chains. The "volume" penetrating into a specified film may not always be proportional to the surface pressure increment. Because of the various factors which contribute to the surface pressure exerted by a monolayer (the equation of state has kinetic, cohesive, and electrostatic terms), the "volume" of the protein penetrating into the monolayer will be related to the surface pressure increment by a factor which will depend on the force area curve of the lipid film and any specific interactions between the components. It is, however, possible to have penetration of a protein into a monolayer without observing an increase in surface pressure such as, for example, if the interaction between protein and lipid results in a condensation of the film which counteracts the increase in pressure due to the penetration of the protein.

Studies of the interaction of radioactivity-labeled albumin and cytochrome c with monolayers of phospholipids has shown that three different types of interaction occur.[248] At low surface pressures, protein molecules in their entirety enter gaps in the expanded lipid films and occupy spaces equivalent to that of the completely unfolded protein at the air-water interface. Further increases in surface pressure were believed to be due to either protein molecules entering the surface layer in a less unfolded form or, alternatively, only part of the protein molecule, possibly a hydrophobic region, penetrating the film. A third type of interaction was an adsorption to the polar surface of the phospholipid monolayer with no change in pressure of the film. The number of protein molecules penetrating the film has been found to depend on the initial pressure of the monolayer for a number of proteins including cytochrome c penetrating phospholipid monolayers,[249,250] $\alpha$-globulin[251] interacting with dihydroceramide lactoside films, and the apoprotein of high density plasma lipoprotein penetrating phosphatidylcholine monolayers.[252] Studies of the penetration of bovine serum albumin into saturated, 1,2-diacylphosphatidylcholine monolayers has shown that the highest initial pressure that protein was able to penetrate increased with increasing hydrocarbon chain length of the phospholipid.[253] Specific interaction of myelin basic protein with different lipids has also been studied at the air-water interface[254, 255] and on protein-lipid complexes.[256] The myelin basic protein was found to have the greatest affinity for cerebroside sulfate, a lipid found most abundantly in myelin. Measurements of the surface radioactivity, using $^{131}$I-labeled basic protein, showed that the amount of basic protein bound to cerebroside sulfate monolayers parallels the pressure increase. Neutral lipids such as lecithin, cholesterol, and cerebrosides showed markedly less affinity for the basic protein. The general conclusion was that electrostatic forces predominate in the interaction between basic protein and lipids, but that hydrophobic interactions are also involved. The authors were able to identify those parts of the protein molecule that are particularly involved in the interaction by peptide mapping the fragments resulting from the proteolytic treatment. They showed that the N-terminal part of the protein molecule (positions 20 to 113) was preserved intact, presumably because it was protected against the action of the proteolytic enzymes by penetration into the lipid phase.

The electrostatic interactions between protein and monomolecular films appear to be most favorable when the net charge between the components is opposite. Thus, when monolayers of phospholipids are ionized and proteins are below their isoelectric point, protein adsorbs strongly to the film and penetration into the hydrocarbon region of the monolayer is facilitated. The digestion of monomolecular films of phospholipids by specific phospholipases has been used as a model system to investigate these electrostatic effects.[257] In these experiments, demonstration of enzyme activity implies that the enzyme has approached the phospholipid-water interface where its active center has become stereochemically oriented about the substrate forming an enzyme-substrate

TABLE 15

**Electrostatic Requirements for Interaction Between Phospholipase Enzymes and Phosphatidylcholine Substrates**

| Enzyme | Activators | Inhibitors | Particle mobility for maximum activity μm/sec/V/cm |
|---|---|---|---|
| Phospholipase A | Ether, $Ca^{2+}$ | — | Not directly related |
| Phospholipase B (penicillium notatum) | Anionic amphipaths $Fe(CN)_6^{3}$ | Cationic amphipaths $Ca^{2+}$ $Mg^{2+}$ $(UO_2)^{2+}$ | −1.7 |
| Phospholipase C (Cl perfringins) | Cationic amphipaths $Ca^{2+}$, $Mg^{2+}$ $(UO_2)^{2+}$ | Anionic amphipaths $Fe(CN)_6^{3-}$ | +0.5 |
| (Rat brain)[a] | Cationic amphipaths $Ca^{2+}$ (not $Co^{2+}Ni^{2+}Mn^{2+}$) | — | — |
| Phospholipase D | Phosphatidic acid $Ca^{2+}$ (not $Mg^{2+}$) | Anionic amphipaths without $Ca^{2+}$ | Negative |
| Triphosphoinositide phosphomonoesterase[b] | Cationic amphipaths divalent ions | Anionic amphipaths | −18 |

[a] Phosphatidylinositol substrate.
[b] triphosphoinositide substrate.

Data from References 258 and 259.

complex. The demonstration of a phospholipase reaction also means that the enzyme protein has initially reacted or complexed with the organized lipid structure in such a way that any deformation of the protein brought about by electrostatic interactions on the substrate surface has not been sufficient to affect the enzyme activity. The electrostatic conditions and divalent ion requirements of a number of phospholipase enzymes are presented in Table 15.

A more recent application of phospholipases to probe membrane structure has been developed by van Deenen and co-workers.[260] They compared the ability of purified phospholipases to attack monomolecular films of varying pressures with their activity on erythrocyte membranes and obtained information relating to the state of compression of phospholipid molecules in the membrane. Phospholipase enzymes from a variety of sources can be separated into two distinct categories; one group readily hydrolyzes phospholipids of intact erythrocytes whereas the others do not. The same distinction was observed with regard to ability of phospholipases to attack monomolecular films of choline phosphatides at different surface pressures. Thus, phospholipase enzymes that were unable to digest the phospholipids of intact erythrocytes also failed to attack monomolecular films of substrate maintained at an initial surface pressure greater than 31 mN $m^{-1}$. Those enzymes capable of hydrolyzing erythrocyte phospholipids, on the other hand, also digested substrate monolayers maintained at initial surface pressures greater than 31 mN $m^{-1}$ but were inactive against films of initial pressure greater than 34 mN $m^{-1}$. It was concluded that the packing of phospholipids at the exterior layer of the intact erythrocyte membrane is equivalent to a lateral surface pressure of between 31 and 34 mN $m^{-1}$.

The properties of the surface layer of membranes are believed to provide a particular environment for certain enzymic reactions. Functional interactions of enzymes with membranes in the control of metabolic reactions have been postulated, for example, in the case of enzymes involved in glycolysis in erythrocytes.[261,262] The association of glyceraldehyde-3-phosphate dehydrogenase with human erythrocyte membranes appears to be specific and reversible and depends on the pH and ionic strength of the medium used for isolation.[263] Furthermore, binding of the enzyme is influenced by

micromolar concentrations of $NAD^+$, NADH, and glyceraldehyde-3-phosphate. The kinetics of binding of $^{14}C$-carboxymethylated glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle to condensed monomolecular films of acidic phospholipids showed that the binding took place by multiple point electrostatic interactions and that binding was markedly influenced by surface charge density and the presence of divalent counter ions such as $Ca^{2+}$.[264] Subsequent studies of the enzymic properties of the protein showed that adsorption to a charged surface considerably modified the kinetic properties of the enzyme compared to that observed in free solution.[265] Recently, studies of the binding characteristics of aldolase to band 3 glycoprotein of human erythrocyte membranes has been reported.

Further evidence of electrostatic interactions between lipid, and proteins has been obtained from studies of artificial bilayer lipid membrane systems. The interaction of cytochrome c with black lipid films, for example, has been studied by Steinemann and Läuger.[266] They found that at low ionic strength, about $10^{13}$ cytochrome c molecules/$cm^2$ are bound to the surface of bilayers of phosphatidylinositol, and these authors concluded that the interaction was mainly electrostatic. The fast desorption of the protein after a rise in ionic strength showed that the protein did not penetrate appreciably into the lipid bilayer, but was located largely on the outside surface of the structure. This is consistent with the X-ray studies of Blaurock which indicate that cytochrome c binds almost entirely to the polar groups of phospholipids. Black lipid film techniques have also been used to study the interaction of tetanus toxin with mixed phosphatidylcholine ganglioside bilayers.[267] When toxin binds to the bilayer, the thickness of the film increased from 7.9 to 11.9 nm, which suggests that the toxin adsorbs to the surface in an unaltered conformation and does not unfold to form an expanded layer over the lipid surface, as was reported with erythrocyte ghost protein.[268]

Interaction of proteins with the hydrocarbon residues of phospholipid bilayers or the effect of protein binding to the structure on the properties of the hydrocarbon region have been investigated by calorimetry and changes in the permeativity to various solutes. Differential scanning calorimetry, for example, has been used to investigate the effect of protein-lipid interactions on thermotropic phase transitions of the lipid acyl chains. The transition temperature of a cytochrome c-phospholipid complex was found to be about 30°C, compared with a transition temperature of 40°C for the pure phospholipid dispersion.[269] The interaction of cytochrome c thus appears to make the phospholipids more fluid. Similar effects on the main lipid transition temperature of aqueous dispersions of *Acholeplasma laidlawii* phospholipid extracts on interaction pwith cytochrome c[270] and complexes formed between phosphatidylserine from ox brain and several basic proteins[271] has been reported. The interaction of the proteins with phospholipids reduced the transition temperature by between 3 and 7°C, and this was reflected in a decrease in the energy barrier for reorientation of a spin-label in the hydrocarbon phase. Papahadjopoulos et al.[272] have studied the interaction of a variety of soluble and membrane proteins with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol by calormetry.

Interaction of protein with the hydrocarbon region of lipids in aqueous dispersions has been shown to affect the permeability characteristics of the bilayer. Thus, proteins extracted from erythrocyte membranes with Triton X-100 appear to enhance the permeativity of phosphatidylcholine vesicles towards sulphate anions.[273] The detergent extracts were considerably enriched in the band 3 component of 95,000 mol wt thought to play a role in anion permeation[17,18] The evidence was derived primarily from studies with disulphonic stilbenes which are potent and specific inhibitors of anion transport in erythrocytes. One such compound, 4,4′-diisothiocyano-2,2′-stilbene disulphonate, binds almost exclusively (95%) to proteins migrating in the band 3 region, and if Triton X-100 extracts from treated cells were added to lecithin vesicles, no increase in anion

permeability was observed. On the basis of this evidence it was suggested that the 95,000 mol wt protein was the membrane component responsible for the enhanced anion fluxes observed in red blood cells.

Spectrin, another major protein isolated from erythrocyte membranes, when added to multilamellar liposomes bearing either negative or positive surface charges, is also found to produce a two- to five-fold increase in the permeativity of glucose.[274] The protein is equally effective in increasing the glucose permeativity of negatively or positively charged liposomes at neutral pH, a finding suggesting that hydrophobic interactions may be involved. In another study,[275] spectrin added to both acidic (phosphatidylserine) and zwitterionic (phosphatidylcholine) phospholipid vesicles at pH values less than the isoelectric point of the protein (pH 3.5) increased sodium permeativity some 100- to 1000-fold; the effect at neutral pH (7.4) was minimal. In the presence of $Ca^{2+}$, however, spectrin produces a substantial increase in the $Na^{+}$-permeability of acidic phospholipid membranes at both neutral and acid pH. The nature of this interplay between protein and calcium is unclear, but a similar synergistic effect with $Ca^{2+}$ and a protein isolated from nerve membranes has been reported.[276] Kinsky[277] and his co-workers have incorporated a number of antigens into multilamellar liposomes and extensively studied the effects on antigen-antibody interactions on increasing the permeability of such liposomes, in the presence of complement.

## B. Reconstituted Membrane Systems

Selective or partial extraction of lipids with nonpolar solvents from a variety of cell membranes often results in a loss of biological activity. The partial restoration of this activity by reintroduction of lipids provided evidence for the functional interdependence of some of the lipid and protein components of membranes. With the development of more refined methods of isolating membrane-bound proteins from their associated lipids, it was soon recognized that in many instances only certain types of lipids were capable of restoring full biological activity of particular proteins; some proteins displayed certain preferences with respect to the hydrocarbon constituent of the lipid whereas in others the lipid class appeared to be the dominant factor. The digestion of lipids with specific phospholipase enzymes, the addition of drugs which compete with the membrane proteins for binding to lipid, and the manipulation of the lipid composition — and hence the fluidity characteristics of membranes — are among other methods used to study protein-lipid interactions. Lipid-dependent enzyme systems are described in detail in a number of reviews,[278-282] and a selection of such systems is presented in Table 16. We shall now examine some of the systems used to demonstrate the role of membrane lipids on the function of particular membrane proteins and multienzyme complexes.

### *1. Simple Extraction and Reaggregation*

These types of experiments are based on the work of Jacobs and Sanadi[283] who depleted intact mitochondria of cytochrome c by extraction with 0.15 *M* KCl and found they could restore oxidative phosphorylation by simply adding cytochrome c to the extracted mitochondria suspended in a suitable buffer. Apart from selective removal of membrane proteins, other methods have been designed to selectively extract membranes of certain lipid components or cholesterol. The reason why such procedures aim to partially or, preferably, to selectively extract particular lipids, is that more drastic methods of delipidating membranes frequently cause protein denaturation and irreversible loss of protein functions. Among the earliest of the successful experiments reported were those of Lester and Fleischer,[284] who described a method for extracting lipids from mitochondria with aqueous acetone and using micellized phospholipids in reaggregation studies. The criterion used for reaggregation was the reactivation of an

TABLE 16

Some Lipid-Dependent Enzyme Systems and Functions

| Enzyme commission number | Enzyme activity/function | Lipid requirement demonstrated |
|---|---|---|
| | Mitochondrial electron transport | Total mitochondrial lipid |
| 1.1.1.30 | D(−) β-hydroxybutyrate dehydrogenase | Phosphatidylcholine |
| 3.6.1.3. | $Na^+$-$K^+$-ATPase | Phosphatidylserine, phosphatidylglycerol |
| 3.1.3.9. | Glucose-6-phosphatase | Phosphatidylethanolamine |
| 1.3.99.3 | Stearyl coenzyme A desaturase | Phospholipid, triglyceride and fatty acid |
| 3.1.3.4. | Phosphatidic acid phosphatase | Total microsomal lipid |
| 3.6.1.3. | $Ca^{2+}$/$Mg^{2+}$ ATPase | Lysolecithin, lecithin, neutral detergents |
| | $Ca^{2+}$ transport | Lysolecithin, lecithin, phosphatidic acid |
| 1.6.99.3 | NADH-cytochrome c reductase | Lecithin:lysolecithin, 1:1 |

enzyme system, and this was achieved by demonstrating the restoration of respiratory activity in lipid-depleted mitochondria.[285] Detailed studies using different lipids to reactivate mitochondria showed that various phospholipid fractions were active, although cardiolipin was effective at lower concentrations than phosphatidylcholine. The rate of binding to lipid-depleted mitochondria was also found to depend on the fatty acid composition of the phospholipids;[286] phospholipids containing a preponderance of polyunsaturated fatty acids such as arachidonic acid bind more slowly but are more firmly retained than are phospholipids containing more saturated fatty acid residues. Aqueous dispersions of saturated phospholipids like dipalmitoylphosphatidylcholine or phosphatidylethanolamine do not restore succinoxidase activity of acetone-extracted mitochondria at 30°C but are effective at temperatures greater than 40°C; dimyristoylphosphatidylcholine, on the other hand, is effective at 30°C but not at 20°C.[287] In this case the specificity is apparently determined by the physical state of the phospholipids. Any phospholipid, provided that functional assays are performed at temperatures above the main lipid transition temperature, $T_c$, appears effective in restoring mitochondrial respiratory activity. More detailed studies have shown that phospholipids are required for each segment of the respiratory chain both in intact mitochondria and in purified systems and for a variety of enzymic activities not only of mitochondria, but for enzyme complexes located in the endoplasmic reticulum and other membranes.[288]

Selective lipid extraction of membranes prepared from brain has been shown to affect the activity of adenylcyclase.[289] Treatment of membranes with ether, a procedure that preferentially extracts the least polar lipids, was found to have a minimal effect on hormone activation of adenylcyclase compared to selective extraction of the more polar phospholipids from liver membranes with an ether; butanol solvent which almost completely abolishes hormone activation of the enzyme and substantially reduces the basal adenylcyclase actvity as well. Specificity of phospholipids concerned in basal and hormone-responsive activities of adenylcyclase has been examined by Rethy et al.[290] in phospholipase-treated or solvent-extracted liver plasma membranes. It was found that ether:butanol extraction reduces basal adenylcyclase activity and responsiveness to

hormones and fluoride but that both activities could be restored by adding specific lipids to the extracted membranes. Thus, complete recovery of basal activity was obtained in the presence of phosphatidylinositol, but hormone and fluoride activation was not affected. Almost complete restoration of adrenaline activation and recovery of some glucagon and fluoride sensitivity could be brought about by the addition of phosphatidylserine. These and other observations suggest that interaction of specific membrane phospholipids with different components of the adenylcyclase enzyme is required in the formation of an active hormone-receptor complex and also to modulate the basal activity of the catalytic site.

*2. Membrane Solubilization*

A second category of experiments is one in which membranes, which have been "solubilized" by, for example, detergent treatment, are made to reaggregate. In many instances, this has been achieved by dialyzing away the detergent to form membrane-like structures easily recognizable in electron micrographs. This type of reconstitution has been observed with inner and outer mitochondrial membranes, microsomal membranes, and bacterial membranes. The capacity for membrane formation is retained by the purified membrane-enzyme complexes. The mitochondrial electron transfer components, for example, can be maintained in a dispersed state in the presence of bile salts, and when the concentration of bile salts in the suspending medium is reduced, the enzyme complexes become less soluble and aggregate to form vesicular structures. Purified preparations of cytochrome oxidase have been used in this type of experiment.[291] When cytochrome oxidase was depleted of lipids, the preparation aggregated after removal of residual bile salts without forming membranes; membrane formation in the lipid-depleted preparation could be induced by adding back mitochondrial phospholipids. Similar reconstitution-type experiments have been described for membranes of *Micoplasma laidlawii*.[292] Membranes are first treated with detergents such as sodium dodecyl sulphate to solubilize the membrane components, and then the detergent is removed by exhaustive dialysis. The removal of detergent causes membrane-like structures to form.

A major disadvantage of this method is that the complete removal of detergent from these systems is very difficult to achieve. Some components bind detergent much more avidly than others, and the presence of detergent-protein complexes in the mixture often affects restoration of biological functions.

*3. Reconstitution from Isolated Components*

Reconstitution experiments involve a separation and purification of the isolated lipid and protein components before reconstitution. (Reconstitution is reserved for those experiments where purified lipids and proteins are recombined to form a functionally active complex although not necessarily oriented in a vectoral manner.) Hatefi et al.,[293] King[294] and King and Takemori;[295] independently reported the reconstitution of a part of the mitochondrial electron transfer chain from the component complexes. Complex I catalyzes the oxidation of NADH by coenzyme Q and Complex III, the oxidation of reduced coenzyme Q by cytochrome c. If these two complexes were merely mixed together and examined immediately, no oxidation of NADH by cytochrome c was observed. However, if the two complexes were first treated with bile salts before mixing, the resulting particle was found to have the capacity to perform the overall reaction which neither of the original complexes in the mixture possessed. The emergence of a new activity was achieved by the interaction of two particles and was presumably facilitated by the residual detergent present in the preparations. This type of experiment constituted the major emphasis of early work of attempts to reconstitute an intact respiratory chain.

A different — and in many respects a more profitable — approach has been to isolate the components of the oxidation chain and to reconstitute those segments with oxidative phosphorylation properties. Such experiments have been described by Racker and Kandrach,[296] who have succeeded in reconstituting the third site of oxidative phosphorylation from purified components. The procedure developed for the reconstitution of biologically active phospholipid vesicles consisted of solubilization of hydrophobic proteins in cholate or deoxycholate and recombination with mixtures of phospholipids that had been exposed to sonic oscillation in the presence of 2% sodium cholate. After removal of cholate by dialysis or gel filtration, vesicular structures with biological activity were formed. By this procedure Racker's group reconstituted other functional activities of mitochondria including the $^{32}$Pi-ATP exchange process, the mitochondrial proton pump,[297] and cytochrome oxidase vesicles exhibiting respiratory control.[298] In some systems, exposure of solubilized complexes to detergent or prolonged dialysis (e.g., the second site of oxidative phosphorylation) prevented reactivation on addition of phospholipids. An alternative method that avoids the use of detergents consists of suspending dried phospholipids in salt solution containing membrane proteins and exposing the mixture to sonication. Apart from those systems reconstituted by detergent methods, a rutamycin and uncoupler-sensitive $P_i$-ATP exchange process has been successfully reconstituted by this procedure. Several new procedures for extraction and reconstitution have recently been devised in which membrane proteins are extracted with silicotungstic acid or are reconstituted by incubation with sonicated liposomes containing 10% lysolecithin.[299,300]

In all such reconstituted systems a purified membrane protein is incorporated into the phospholipid vesicles. Almost invariably it has been found that an optimum mixture of phospholipids is required to reconstitute the various mitochondrial systems. Thus, not only is the addition of certain phospholipids necessary, e.g., phosphatidylcholine and phosphatidylethanolamine, but they must be added in a specific ratio. It seems that the permeability to protons, for instance, and the formation of closed vesicles are important factors that depend on the composition of phospholipids.[301] Furthermore, these reconstituted systems appear to be somewhat different from the complexes of the original membrane. For example, not all the protein interacts with the phospholipid bilayer, and some of it may be only partly inserted into the lipid structure. Even the position of the inserted protein is not the natural one. Moreover, the univectorial assembly observed in the mitochondria is randomized in reconstituted systems since the protein molecule is oriented equally in both directions across the lipid bilayer.

In addition to studies of complexes in mitochondria, a number of individual enzyme components have been isolated from mitochondrial membranes; and in reconstitution studies, dependence on specific phospholipids for enzyme activity has been demonstrated. One of the first enzymes to be investigated in this way was β-hydroxybutyrate dehydrogenase of beef heart mitochondria.[302] The enzyme could be reconstituted with mitochondrial lipids or lecithins from a variety of sources to form an active enzyme-lipid complex.[303-305] Although the enzyme appears to have an absolute requirement for lecithin when phosphatidylethanolamine is added together with phosphatidylcholine, reactivation of the enzyme is enhanced[306] and the effect appears to be related to the hydrocarbon chain composition of the phosphatidylethanolamine.[307] Recently the lipid specificity of β-hydroxybutyrate dehydrogenase activation has been studied with a variety of synthetic phospholipids.[308] It has been shown that a hydrophobic chain followed sequentially by a negative and a positive charge, as in stearylphosphorylcholine, is the minimal structural requirement of an activator. However, the stability of the complex depends on the aggregation state of the activators, complexes of appreciable stability being formed only with those phospholipids ($C_9$ to $C_{18}$) which exist in bilayer

membrane-like structures. Lecithins with saturated and unsaturated fatty acid chains activated the enzyme, but the latter formed somewhat more stable complexes. The strength of interaction between the protein and phosphatidylcholine decreases at the main lipid phase transition temperature. It is clear that the enzyme has an unusual specificity for both the ionic groups and the fatty acid side chains.

Apart from mitochondrial electron transport complexes and enzymes, reconstitution studies have been reported for a number of other membrane proteins including $Ca^{2+}$-activated ATPase of sarcoplasmic reticulum.[301] and the proton pump of *Halobacterium halobium*.[309] Bacteriorhodopsin has been incorporated into both black lipid membranes and lipid vesicles.[310-315] Demonstration of a functional reconstitution in many of these systems is not possible because asymmetric incorporation into sealed vesicles is required to demonstrate vectorial light-driven proton translocation (no method has yet been devised that would selectively inhibit the proton pump from one side of the membrane, a procedure that would be necessary to observe pumping activity in a symmetrically reconstituted system). Asymmetric incorporation of bacteriorhodopsin into lipid membranes has been achieved by cosonicating isolated purple membrane with dimyristoylphosphatidylcholine, and proton pumping in this system was observed at temperatures both above and below the temperature at transition (Tc).[316] The bacteriorhodopsin is dispersed and aggregated, respectively, under these conditions[315] suggesting that segregation within the purple membrane patches is not required for the functional integrity of the protein.

Reconstitution of cholate-solubilized $Ca^{2+}$-activated ATPase of sarcoplasmic reticulum with synthetic phosphatidylcholines with defined fatty acid composition has been reported and the effects on activity of the enzyme noted.[317] The activity of the ATPase appeared to depend on the hydrocarbon chain of the phospholipid and, at 30°, greatest activity was restored by reconstitution in dioleoyllecithin > dimyrstoyllecithin > dipalmitoyllecithin; dilauroyllecithin inactivated the enzyme. Since there was no apparent relationship between the phase transition temperature (Tc) of the bulk lipid and the activity of the enzyme, it was suggested that the lipid in immediate contact with the protein (numbering 30 to 35 molecules per ATPase in all) the "so-called" lipid annulus had unusual properties endowed by this contact with the protein.[318] Indeed, experiments were presented which purported to show that the lipids of this annulus were selected and that cholesterol, which is present in the sarcoplasmic reticulum membrane, is specifically excluded from contact with the active enzyme.[319]

Reconstitution of the visual pigment, rhodopsin, has been reported, but as yet the precise functional interaction between the protein and the retinal rod outer segment disk membrane is not known. It has been suggested that the interconversion of metarhodopsin I to metarhodopsin II is a useful parameter to establish functional reconstitution since this is believed to involve an interaction between the chromophore, retinal, and the protein moiety, opsin, leading to a change in conformation of the protein and in turn its interaction with lipids in the membrane.[320] Reconstitution of rhodopsin in a variety of unsaturated phospholipids such as egg lecithin, dioleoylphosphatidylcholine, and egg phosphatidylethanolamine all showed first order kinetics for the metarhodopsin I → metarhodopsin II transition with the same rate as that obtained in the disk membranes. On the other hand, the transition rate was considerably slower when reconstitution was performed with short chain saturated lecithins (didodecanoylphosphatidylcholine and dimyristoylphosphatidycholine) even when measured at temperatures where the hydrocarbon chains would be expected to be arranged in a disordered configuration.[321] This would indicate that unsaturated fatty acyl residues are required to produce an efficient metarhodopsin I to metarhopsin II transition and that there is no polar group specificity, at least, capable of distinguishing between posphatidylcholine and phosphatidylethanolamine.

Specific lipid requirements have, however, been demonstrated for the Rh antigenic activity of erythrocytes. The Rh antigen is difficult to extract in an active form from the red cell membrane suggesting that lability is due to its separation from other components of the membrane. Support for this view has been obtained by Green,[322] who reported that specific phospholipids were required to restore antigenic activity to the isolated protein. Both polar and hydrophobic regions of the phospholipid molecules appear to participate in the binding. Thus, phosphatidylcholine and phosphatidylethanolamine were the only phospholipids examined that were capable of restoring Rh antigenic activity, and at least one unsaturated fatty acyl residue was necessary in order to reconstitute the active antigen; phospholipids with completely saturated hydrocarbon chains did not interact specifically with the antigen.

### C. Lipase and Drug Effects

A useful method of demonstrating lipid protein interactions in biological membranes which avoids extraction and possible inactivation of specific membrane components is to treat membranes with specific phospholipase enzymes or to add drugs that form complexes with certain lipids and then examine the effects of these procedures on some functional activity. When beef heart mitochondrial membranes are treated with phospholipase A, for example, the activity of β-hydroxybutyrate dehydrogenase is decreased. This was found to be due to a release of enzyme from the inner mitochondrial membrane in the form of an inactive apoenzyme.[303,323] It was found that the addition of chaotropic agents improved the efficiency of extraction of the apoenzyme from the residual mitochondrial fraction.[324] The apodehydrogenase could be purified from enzyme-treated mitochondria free of detectable lipids (or detergents) and in a water soluble form. The apoenzyme was found to be reactivated by adding sonicated or microdispersed phosphatidylcholine or mixtures of phospholipids containing phosphatidylcholine, suggesting that lipids — and, in particular, phosphatidylcholine — were necessary for enzymic activity.

The treatment of other membranes such as the plasma membrane of different cells with phospholipases has shown that other enzymes are partially or completely inactivated by this procedure. Adenylcyclase from liver plasma membrane is inactivated by phospholipase digestion.[290] The essential role of membrane lipids in the function of $Na^+$-$K^+$ activated ATPase of plasma membranes has also been established on the basis of complete or partial inactivation by digestion of membrane phospholipids with phospholipase A[325,326] or phospholipase C[327,328] as well as treatment with nonionic detergents,[329] glycerol,[330] or organic solvents such as ethanol-hexane.[331] Attempts to reactivate this enzyme by addition of specific phospholipids have been made in order to identify specific lipid-protein interactions. However, these questions remain unsolved. A complete reactivation of delipidated preparations can often be achieved by the addition of mixed phospholipid extracts, and phosphatidylserine appears to be the most effective constituent. The absolute dependence of enzyme activity on interaction with phosphatidylserine, however, has been challenged by de Pont et al.,[331] who treated a bovine brain preparation possessing high ($Na^+$-$K^+$)-ATPase activity with phosphatidylserine decarboxylase and converted 99% of membrane phosphatidylserine to phosphatidylethanolamine without loss of enzyme activity.

The binding of certain drugs to particular membrane lipids and the formation of complexes that interfere with specific interactions with membrane proteins has been reported.[203] The interaction between the polyene antibiotic, filipin and cholesterol, and melittin with phospholipids and their effects on adenylcyclase activity has already been mentioned in this context (see Section III.C.2.).

### D. Summary and Critique

Model systems have been particularly useful for providing understanding and insight

into the types of interactions which can exist between the various membrane components. In the case of cholesterol-lipid interactions, there is now considerable information and general agreement about the manner in which the cholesterol molecules can modulate the lipid fluidity. The organization of cholesterol in the plane of the membrane is, however, uncertain, and further work is required to determine whether stoichiometric "complexes" of a 2:1 lipid to cholesterol ratio or a more random structure is the correct arrangement. Related studies of permeability processes through lipid-cholesterol bilayers links the structural studies to transport mechanisms. The effect of cholesterol on lipid phase transitions in the model systems has also been paralleled in certain natural membranes.

Studies of polypeptide-lipid interactions have been useful for modeling expected membrane interactions such as the effect on membrane fluidity caused by electrostatic interaction or, alternatively, hydrophobic interactions. Studies of the manner in which a hydrophobic polypeptide can be squeezed out of the high melting lipid region of a mixed lipid system is also valuable. It provides insight into the way proteins in natural biomembranes can become segregated upon cooling the membrane, and the ideas of packing faults, defects, etc. are useful concepts that arise from these considerations.

Studies of reconstituted systems have also been useful for studying protein-lipid interactions. However, there appear to be a number of problems associated with this technique. These include the difficulty of ensuring, firstly, that all the membrane lipid has been exchanged with the selected pure lipid — in some cases, residual membrane lipid tightly bound to the protein may be carried over into the recombined system, and, secondly, that all detergent has been removed after the recombination process is complete.

Some experiments using these recombined protein-lipid structures give results which are not yet explained, e.g., calcium-activated ATPase enzyme activities show a discontinuity with dimyristoyl lecithin at 23°C (i.e., the transition temperature of the pure lecithin-water system) while the complex with dipalmitoyl lecithin shows discontinuities at about 28°C and 38°C, both of which are lower than the transition for dipalmitoyl lecithin-water system (41°C). What seems clear is that improved characterization of these reconstituted systems is required, e.g., X-ray and electron microscope studies — and sometimes calorimetric methods — to provide a firm basis for the deductions which are made.

When we consider protein-lipid interactions of intrinsic proteins in membrane systems, there remain a number of questions which studies of these systems should provide.

1. Do some membrane lipids remain specifically bound to these proteins and undergo no or limited exchange with the remaining membrane lipids?
2. Do membrane lipids interact in a more random nonspecific way with those proteins?

What is clear is that any intrinsic protein will perturb its surrounding lipid in an analogous manner to that of cholesterol, i.e., inhibiting some rotational isomers of the chains and stiffening the chains. Some workers suggest, however, that most if not all intrinsic proteins have a single lipid shell termed an "annulus", composed of lipids specifically selected by the protein and into which cholesterol molecules cannot penetrate. It is argued that the lipids of this annulus do not exchange readily with the bulk lipid. The model for these protein-lipid complexes, e.g., $Ca^{2+}$-activated ATPase and cytochrome oxidase consists of lipid molecules tightly packed around the circumference of the protein, i.e., it has maximum coordination number (for the cytochrome oxidase system the term used for this adjacent lipid is boundary layer lipid). In order

to achieve lipid-protein ratios low enough to inhibit catalytic activity, it is likely — and evidence is, in fact, available to show — that the properties associated with the bilayer arrangement of lipids is lost. Thus, difficulties in packing such lipoprotein complexes into planar membrane structures can be envisaged, and the effects of such arrangements on enzyme activity are unknown.

In short, various terms now exist in the literature to describe these lipid-protein interactions which are sometimes used in a synonymous way but which may correspond to different effects. These include residual lipid, boundary layer lipid, annulus lipid, and halo lipid. This confusion of terms requires further careful analysis. There may well be specific lipid-protein interactions in certain membranes and nonspecific interactions in other membranes. The situation at present is confused, and no clear generalization exists apart from the obvious perturbation of the surrounding fluid lipid which must be caused by any intrinsic protein.

## V. MOLECULAR ORGANIZATION IN CELL MEMBRANES

The formulation of models of membrane structure have served in the past as reference points for experimental investigation of biological membranes. Many of these models were based substantially on information obtained from plasma membranes of the myelin sheath and erythrocytes. The membrane of myelin, in particular, contains a high proportion of lipid relative to the protein constituents, and consequently the structure tends to be dominated by the lipid. The reverse situation is the case with mitochondrial membranes, where there is substantially more protein than lipid. Many attempts have been made to generalize the structures of all cell membranes into a single, simple, model structure. This in some cases has led to confusion rather than clarificaion of the situation because the detailed molecular structure of biological membranes varies considerably in complexity from one membrane type to another. We will examine those features that appear to be common to cell membranes in general and discuss some of the distinctive characteristics of particular cell membranes.

### A. Lipid Bilayer Structure in Cell Membranes

More than 50 years have elapsed since Gorter and Grendel[335] proposed that a phospholipid bilayer constituted the basic structure of the erythrocyte membrane. They began with the premise that membranes were, in part, lipoidal in character, since hydrophobic solutes were known to penetrate membranes more easily than hydrophilic molecules.[336] There was also great interest in the surface-active properties of membrane lipids which had been shown to orient at oil-water and air-water interfaces in a predictable manner.[337] Their experiment consisted simply of extracting the lipid from a known area of erythrocyte membrane and measuring the area occupied by the lipid when spread as a monomolecular film at the air-water interface. This area of the film was almost twice that of the membrane from which the lipids were extracted, so they concluded that the membrane consisted of a two-dimensional array of lipids 2 molecules thick. The original experiments have been reexamined more recently by Bar et al.,[338] who identified certain technical errors in the method. For example, calculation of the area of membrane from which the lipids were extracted was underestimated, but this was compensated to a large extent by an incomplete extraction of the lipids. A more serious problem, however, was an uncertainty of the precise packing density of the lipid molecules in the monolayer, which we now know from X-ray and other measurements to be fairly close-packed. When a complete lipid extract of erythrocyte membranes is spread at the air-water interface, the minimum area occupied by the film is about 140% of the total membrane area, and thus no *a priori* evidence is obtained to indicate a bimolecular arrangement. Similar experiments have now been extended

TABLE 17

**Estimates of Membrane Surface Area Assumed to Consist of Lipid Bilayer Calculated from the Limiting Area Occupied by a Total Membrane Lipid Extract Oriented as a Monomolecular Film at an Air-Water Interface**

| Membrane | Ratio protein: total lipid (wt/wt) | Maximum membrane surface as lipid bilayer % |
|---|---|---|
| Plasma membranes | | |
| Myelin | 0.28 | 103 |
| Erythrocyte | 1.50 | 67 |
| *Acholeplasma laidlawii* | 1.78 | 62 |
| Endoplasmic reticulum | 0.90 | 83 |
| Sarcoplasmic reticulum | 1.00 | 80 |
| Mitochondrial membranes | | |
| Inner membrane | 3.55 | 40 |
| Outer membrane | 1.22 | 72 |

to other cell membranes for which the necessary chemical data are available (Table 17) and, in general, the surface area of the close-packed lipid monolayer from a certain area of membrane is inversely related to the protein content of the membrane and, with the exception of myelin, is always less than twice the area of the membrane.

A different approach using the erythrocyte membrane as a model has been applied by Engelman,[339] in which assumptions concerning the degree of compression of monomolecular films in surface area measurements are to some extent avoided. Firstly, he calculated the volume of membrane occupied by the hydrocarbon residues of phospholipid molecules, assigning an average length of 16.5 carbon atoms to acyl hydrocarbon chains extending into the hydrophobic region, and a mean of 1.26 unsaturated bonds per chain. To this he added the volume of cholesterol, obtained from density measurements, assuming that the entire molecule resides in the hydrophobic region of the membrane. The value obtained was too large for the membrane to consist of a single layer of lipid molecules yet insufficient by 10 to 20% to cover the entire red cell surface twice; hence, again no direct evidence is obtained for a membrane bilayer. If a bilayer of lipid is postulated, then it must be further assumed that the discrepancy in the calculation must arise from intepolation of nonlipid components into the membrane structure.

A slightly more reliable method of establishing the presence of two layers of phospholipids in biological membranes is to selectively remove these lipids by phospholipase digestion and then correlate the amount of phospholipid removed from the structure with the reduction in membrane surface area. Treatment of human erythrocyte membranes with phospholipase c, which converts phospholipids into diglycerides and water soluble products, has been attempted with this objective.[340] It was found that approximately 70% of the total membrane phopholipids were susceptible to hydrolysis by phospholipase c, and the product accumulated in the form of small droplets of diglyceride which remained associated with the membrane. When digestion of susceptible phospholipids was complete the surface area of the membrane was reduced to about half the original area, suggesting that membrane lipid exists in at least two layers and that the residual membrane consists of about 25% of nonlipid components. It is interesting that no apparent morphological changes were observed when thin sections of the enzyme-treated membrane were examined by electron microscopy. Freeze-fracture replicas of partially digested membrane preparations showed a direct correlation between the density of membrane-associated particles visible on the inner membrane

fracture faces and the amount of phospholipid hydrolyzed up to a point where about half the total membrane phospholipid had been digested. More extensively digested membranes showed few membrane cleavage planes, and no reliable measurements could be performed in these preparations. These results are also consistent with the supposition that membrane-associated particles are unlikely to consist of phospholipid since the number of particles was unchanged after phospholipase treatment.

Direct physical measurements such as X-ray diffraction have been used to examine the structure of biological membranes. Membranes that exist in multilamellar arrays lend themselves well to X-ray studies and considerable detail has been obtained from myelin, retinal rod outer segment disc membranes, and chloroplast thylakoids. Blaurock and Worthington[341] obtained X-ray diffraction patterns from a variety of periferal nerve and central nervous system myelin in which discrete diffraction lines were observed out to 11 orders of diffraction. Diffraction orders greater than five, however, were usually of weak intensity partly due to the cylindrical shape of the specimens and partly due to the membrane structure itself. The radial repeat distances of periferal nerve myelin (17.9 to 18.2 nm) was found to be greater than central nervous system myelin (15.3 to 15.9 nm). Different intensity distributions for diffraction orders 1 to 5 between periferal nerve and central nerve myelin were also noted, but these differences were qualitatively similar in myelin from different sources including frog, chicken, and rat. Detailed X-ray studies of swelling experiments with frog sciatic nerve myelin were reported,[342] and in sucrose, for example, low-angle diffraction patterns were obtained with up to 13 diffraction orders of a repeating unit d = 38.8 nm. Worthington et al.[344,345] have constructed models for the structure of myelin and compared the theoretical diffraction pattern from such structures with that obtained experimentally. The system was based on a simple step function and is defined by a number of parameters determining, in the one-dimensional case, the limits of regions of differing electron density, these regions being identified with different parts of the double-membrane system. The parameters are then adjusted and refined until optimum agreement is achieved between the predicted intensities and those obtained experimentally. An obvious prerequisite for this procedure is that the number of diffraction parameters exceed the number of model parameters being refined. The results from sciatic nerve indicate a thickness for a single nerve membrane of 7.5 nm: the membrane is asymmetric with a narrow central region (2.1 nm) of low electron density. The asymmetry of the nerve membrane unit arises primarily through the presence of an electron dense layer 1 nm thick which faces the extracellular fluid face. Other studies of this membrane system have been reported[346, 347] in which a heavy atom labeling method has been used to provide a solution for the phasing of myelin structure amplitudes discussed previously in connection with X-ray studies of lipid-water systems. The method involved titrating osmium tetroxide, platinic chloride, or potassium permanganate into the nerve. In the case of the osmium label, the Patterson function indicated that the metal interacts with a single site when administered in small doses, but with heavy labeling a second site can be identified or, alternatively, there is an extensive reorganization of the membrane components. Computer analogue techniques were used to calculate the electron density distribution of heavy-atom labeled membranes, assuming one or two site deposition of the metal, and these were compared with the electron density distribution of the native structure. The resuts obtained differed from conventional X-ray electron density profiles, particularly in the presence and location of peaks of high electron density at the two centers of symmetry. Other X-ray studies of nerve myelin have been reported in which diffraction data giving an effective point resolution of 0.7 nm has been used to construct a model of the molecular organization within the membrane.[348] The model consisted of a lipid bilayer with cholesterol intercalated between the hydrocarbon chains of the phospholipids, but such models are only con-

sistent with the X-ray data. Similar arguments apply to models of other regularly oriented membrane systems such as retinal rod outer segment disc membranes and chloroplast membranes constructed from information derived from X-ray diffraction data.[352]

A number of isolated membrane systems have also been investigated by X-ray diffraction techniques. Erythrocyte membranes stacked artificially by centrifugation and dehydrated to an extent of 10 to 20% by weight of water give three or four lamellar spacings corresponding to a repeat distance of 10 to 12 nm.[353, 354] This diffraction pattern was produced only when essential water of hydration is present since changes in the diffraction pattern result from further dehydration of the membrane preparation. An interpretation in terms of a lipid bilayer with nonlipid components located on the surface was suggested as being quantitatively consistent with the observed intensity distribution in the diffraction pattern. A model of erythrocyte membrane structure similar to this was favored at the time. X—ray scattering from dispersions of hemoglobin-free erythrocyte membranes contaning about 5% solids has been reported by Wilkins et al.[355] The corrected scattering pattern consists of a broad band with an intensity maximum at 4.5 nm and a weak band at 1.5 nm, and in overexposed films a band at about 2.2 nm is just discernible. Similar patterns were also observed with sonicated preparations of phospholipid and interpreted from electron density distributions derived from multilamellar arrangements of lipids[356] to indicate the presence of a bilayer orientation of the membrane lipids. Thus, scattering curves of the type obtained from erythrocyte suspensions were taken to represent the presence of extensive — although not necessarily continuous — phospholipid bilayer-type structures in these membranes. The presence of cholesterol in both lipid dispersions and membranes led to an increased odering of the hydrocarbon chains since this correlated with an increased intensity of the third maximum compared to the second whereas the reverse is true when cholesterol is absent. Low-angle X-ray diffraction patterns have been reported for membranes of *Halobacterium halobium*[357] inner and outer rat liver mitochondrial membranes,[358] guinea-pig intestinal brush border membranes,[359] and plasma membranes of *Acholeplasma Laidlawii*.[360] When the membranes of *Acholeplasma* were enriched with erucate and examined at low temperature, maximum bands were observed at 5.2 nm, 2.6 nm, and 1.7 nm with the second and third bands being of equal intensity. The erucate-enriched membranes give equivalent Bragg spacings which are larger than those for palmitate-enriched (multiples of 4.7 nm approximately) and oleate-enriched (multiples of 4.6 nm approximately) membranes, presumably reflecting differences in hydrocarbon chain lengths of the major fatty acids of the membranes. The equal intensities of the second and third bands was believed to result from a localization of the terminal methyl groups of the hydrocarbon chains in the center of a bilayer arrangement of the membrane lipids. Interestingly, when diffraction spacings are obtained from membranes maintained at temperatures above the phase transition temperature of the lipids, the three bands observed move to a smaller spacing, and the third band appears considerably weaker than the second. These changes have been noted in pure lipid systems and are consistent with a decrease in thickness of a phospholipid bilayer on heating from a low temperature where the hydrocarbon chains are in a hexagonally-packed gel phase to a temperature where the chains are disordered. Furthermore, a decrease in intensity of the third band compared to the second would be expected if the terminal methyl groups of the hydrocarbon chains are not vigorously confined to the central region of the structure.

Neutron diffraction methods have been applied to biological membrane systems and, as expected, qualitatively similar scattering intensities of those of X-rays are observed. Earlier studies of the membranes of mammalian nerve myelin in $D_2O$ (Deuterium Oxide) solution gave only a single intense peak of scattering at an equivalent Bragg

spacing of 9 nm, possibly due to poor instrumentation.[361] Using more refined techniques, seven diffraction orders have been observed in rabbit sciatic nerve in $D_2O$ solution with a periodicity of 18 nm.[362] Using phase assignments as for X-ray diffraction analyses, Kirschner[363] has calculated a neutron Fourier profile of 2.6 nm resolution for myelin. Neutron diffraction has since been used to investigate the bilayer structure of other biomembranes (for review, see Reference 361.).

In general, the X-ray and neutron diffraction evidence from biological membranes is usually consistent with the orientation of at least some of the membrane lipids in a bilayer arrangement. The evidence for this is that from membrane preparations electron density profiles and diffraction patterns from membrane preparations are similar to those observed with phospholipid-water structures where a bilayer arrangement has been established more firmly. The method cannot provide any quantitative information about the overall contribution that lipid bilayer may make to the structure, and other types of lipid arrangement such as hexagonal or cubic phases cannot be entirely excluded as structural components of biological membranes.

Spin-label studies using fatty acid and sterol spin-probes in oriented multilayers of egg lecithin indicate that the probes align with the long axis of the lipid molecule perpendicular to the surface supporting the multilayer samples.[364] The spectral features also display marked changes depending on the orientation of the multilayer structures in the magnetic field of the spectrometer such that the long axes of the probes tend to orient perpendicular to the plane of the multilayer. Demonstrating ordered orientations in biological membranes, as with X-ray diffraction studies, requires multilayer membrane arrays to be most convincing. Canine erythrocytes, for example, oriented by flow methods and containing sterol and fatty acid spin-probes show marked spectral anisotropy in the magnetic field despite the relatively poor alignment of the cells.[365] Taking into account these experimental difficulties, the ordering of the lipid spin labels supports the interpretation that the red cells were oriented with respect to the magnetic field and that the hydrophobic regions accessible to the spin labels tend to be ordered. Assuming the most probable orientations of the cells, this ordered region orients the long axis of the spin-probes perpendicular to the biconcave surface of the cell. Similar spin-label studies of invertebrate nerve fibers also show some anisotropy, with evidence that the long axis of the spin-label tends to orient perpendicular to the cylindrical surface of the nerve bundle. The spin-label studies of biomembrane sytems are compatible with the existence of ordered lipids, such as in multilamellar arrays of phospholipids, that orient into bilayers and thus consistent with the presence of some similar lipid in biological membranes.

The existence of endothermic lipid phase transitions in biological membranes has been observed in a variety of cells. Studies of pure phospholipid systems has shown that endotherms associated with heating a previously cooled sample arise from cooperative "melting" of hexagonally close-packed hydrocarbon chains of phospholipids oriented in bilayer configuration. The appearance of endothermic phase transitions in biomembranes has sometimes been cited as evidence for the existence of lipid bilayer structure in the membrane. It must be emphasized, however, that lipid phase separation and protein aggregation effects (see Figure 11) can accompany the cooling process. The subsequent heating curves observed by calorimetry then correspond to an altered membrane structure, and the endotherms arise from the melting of the lipid from which the (intrinsic) protein has been excluded. Cooling curves are also subject to the same difficulties because reorganization of the structure can occur while the cooling is taking place.

Taken together, the monolayer measurements of lipid surface area, electron microscopic techniques, X-ray and neutron diffraction analyses, and spectroscopic and calorimetric measurements are consistent with an arrangement of lipids in biological

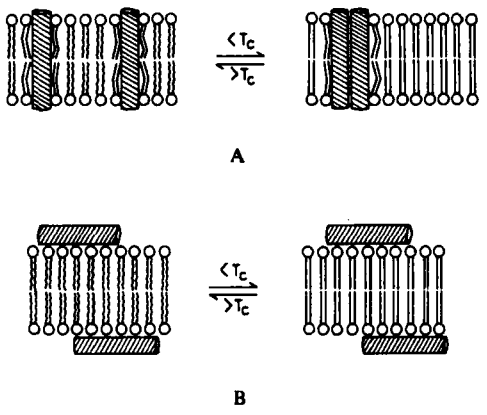



FIGURE 11. Diagrammatic representation of two possible processes underlying the observed endotherms obtained from biomembranes using calorimetric techniques. In (A) the membrane contains intrinsic protein, represented by the shaded cylinders, which tends to perturb the adjacent lipid molecules of the bilayer. Cooling the membranes to temperatures below the phase transition temperature of the lipid ($<T_c$) leads to aggregation of the protein, leaving large areas of crystalline lipid. Upon heating the membrane to temperatures $>T_c$, the crystalline lipid melts, giving rise to the observed endotherm. In (B) the protein is located exclusively on the outside of the bilayer (extrinsic protein), and prior cooling of the membrane to temperature $<T_c$ causes the hydrocarbon chains of the lipids to crystallize. It is the subsequent melting of all the lipid chains that gives rise to the observed endotherms.

membranes corresponding to a bilayer configuration. The proportion of lipid bilayer probably varies greatly between different membrane types depending on the proportion of polar lipids to intrinsic membrane proteins present in the particular membrane.

### B. Asymmetric Disposition of Membrane Lipids

Studies of sonicated dispersions of phospholipid mixtures such as phosphatidylcholine and phosphatidylglycerol[366] and phosphatidylcholine-cholesterol[367] by nuclear magnetic resonance spectroscopy have shown that considerable asymmetry in lipid distribution is generated in the formation of vesicles with low radii of curvature. In the case of mixed phospholipid vesicles, the zwitterionic phospholipid, which is capable of a closer packing arrangement than charged phospholipid, appears to orient on the inner surface of small vesicles, and the acidic phospholipids tend to distribute into the outer leaflet. Likewise, in vesicles consisting of a mixture of phospholipid and cholesterol, a higher proportion of cholesterol is located on the inner surface while the phospholipids with bulky polyunsaturated hydrocarbon chains pack more readily on the outer surface. Such interpretations imply that phospholipid asymmetry could be induced in biological membranes simply in response to changing membrane curvature. Moreover, segregation of lipid components in the plane of the membrane, as well as asymmetry across the bilayer, could be influenced by the folding of the membrane, thereby creating contiguous regions within membranes possessing highly specialized properties. We now examine the evidence for an asymmetric arrangement of lipids in biological membranes.

*1. Phospholipase Enzyme Digestion*

The first indication that phospholipids are not arranged asymmetrically across the red cell membrane was obtained by Turner and his colleagues[368, 369] in studies of the action of phospholipase A on erythrocytes of various species. They found that cobra venom enzyme, which hydrolyzes the fatty-acyl residues of glycerophosphatides, was lytic to most mammalian erythrocytes with the exception of those from ruminant species. The phospholipid composition of ruminant erythrocytes is distinct from those of other species because sphingomyelin largely replaces phosphatidylcholine as the predominant phospholipid class, and this may explain, in part, their remarkable resistance to lysis. In contrast, when isolated erythrocyte membrane preparations from most species, including ruminants, are treated with phospholipase A, they are rapidly depleted of all diacylglycerophosphatides because the enzyme is accessible to both sides of the membrane. This indicates that in ruminants, at least, sphingomyelin is the predominant phospholipid on the outer surface of the membrane. It should be noted that the proportion of diacylphosphoglycerides relative to plasmalogen varies in erythrocytes from species to species and is high, for example, in human erythrocytes compared to those from ruminants.

Enzymic procedures have been considerably refined by using highly purified phospholipases from different sources.[370] Pure phospholipase $A_2$ for example, which removes only the fatty-acyl residue of the glycerol carbon-2 position, is not itself lytic to erythrocytes, but crude preparations of the enzyme often possess additional components which operate in conjunction with the enzyme to produce hemolysis. It is obvious that if the plasma membrane ruptures, then enzyme action is not restricted solely to the outer surface of the membrane, and no meaningful conclusions can be drawn with regard to phospholipid distribution. Roelofsen et al.[371] have shown that purified pancreatic phospholipare $A_2$ and phospholipase C of *Bacillus cereus,* both of which attack glycerophosphatides but not sphingomyelin, are not lytic to human erythrocytes under isotonic conditions, but neither do they produce any significant phospholipid degradation. Furthermore, preliminary treatment of the cells with proteolytic enzymes or neuraminidase is also without effect on the hydrolytic activity of these enzymes. If the membrane is ruptured however, and the cytoplasmic surface of the membrane is exposed to the enzymes, there is an almost complete degradation of the glycerophosphatides, but, as expected, sphingomyelin remains intact. Other sources of phospholipase $A_2$ such as bee and cobra venoms are also nonhemolytic to human erythrocytes under isotonic conditions, but these enzymes hydrolyze about 20% of the total phospholipids, and nearly all of this is phosphatidylcholine. Quantitatively, this represents almost two thirds of the total phosphatidylcholine, all of which is presumably located on the outer surface of the membrane. A rather interesting feature of these experiments is the fact that even though substantial amounts of phosphatidylcholine is converted to lysophosphatidylcholine, the membrane appears to remain intact. The susceptibility of membrane glycerophosphatides to hydrolysis by phospholipase enzymes also appears to be markedly dependent on the ionic strength of the suspending medium.[371] When erythrocytes were treated with purified pancreatic phospholipase $A_2$ and *B. cereus* phospholipase C under hypotonic conditions, for example, there was a rapid and extensive hydrolysis of phospholipids leading to hemolysis of the cells. This may indicate that penetration of the enzymes into the substrate is facilitated in some way by low salt concentration. In agreement with earlier observations, ruminant red cells were surprisingly resistant to hemolysis, and no substantial breakdown of glycerophosphatides could be detected even when the salt concentration was reduced. This result is to be expected if the location of these phospholipids is predominantly on the inner surface of erythrocyte membrane.

In other experiments[372] using a combination of purified phospholipases $A_2$ and C,

it was found that resealed human erythrocyte membranes became leaky after digestion even under isotonic conditions, indicating that manipulation of the membrane associated with ghost preparation causes either a change in the susceptibility of glycerophosphatides to hydrolysis or an alteration of their disposition in the membrane. Finally, Verkleij et al.[373] have reported that only 68% of the phosphatidylcholine of intact human erythrocytes can be degraded by phospholipase $A_2$ *(N. naja)* without hemolysis. No degradation of phosphatidylethanolamine or phosphatidylserine was observed, and phospholipiase C *(B. cereus)* produced no phospholipid degradation in the intact cell. Sphingomyelinase *(S. aureus)*, on the other hand, hydrolyzed 85% of the sphingomyelin of the membrane. When washed ghosts were subjected to digestion by phospholipase $A_2$ and C, total degradation of all glycerophosphatides was observed. These results have been confirmed by Kahlenberg et al.[374] using inside-out vesicles generated from leaky ghosts and subjecting them to digestion with phospholipase $A_2$ and C. The overall conclusion from enzymic dissecttion of phospholipid disposition in red cell membranes is that these compounds are asymmetrically arranged with most of the sphingomyelin, about 70% of the phosphatidylcholine, and some phosphatidylethanolamine located on the outer leaflet of the membrane with the bulk of the acidic phospholipids oriented on the cytoplasmic surface.

There is some evidence that cholesterol is distributed asymmetrically in biological membranes (see below). The use of cholesterol oxidase as a probe of cholesterol disposition in membranes is likely to prove a useful and more direct method to investigate asymmetry of this sterol.

### *2. Phospholipid Exchange Measurements*

Phospholipid asymmetry of rat erythrocytes has also been examined by phospholipid exchange with lipid dispersions. Although the presence of exchange proteins does not accelerate the exchange of phospholipid between intact erythrocytes and liposomes,[173,376] there is a rapid exchange of about 75% of phosphatidylcholine from resealed erythrocyte ghosts, with the remainder exchangeable at a slower rate. This is consistent with the presence of most of the phosphatidylcholine on the outer surface of these membrane preparations. Bloj and Zilversmit[377] examined the exchangeable pool sizes in inside-out vesicles of rat erythrocyte membrane and found that some but not all of the asymmetry was lost in preparation of these vesicles, suggesting that some rearrangement of the membranes had taken place despite the fact that certain asymmetric functions are preserved.[378] The asymmetry of phosphatidylcholine in rat erythrocytes has been examined in relation to the metabolic lability of the phospholipid, and evidence supporting a relatively slow but measurable transmigration of the phospholipid has been reported.[379]

### *3. Chemical Labeling Methods*

Chemical labeling procedures employing reagents specific for amino groups have provided additional evidence of phospholipid asymmetry in erythrocyte membranes. The fluorescent reagent, stilbene-4-acetamido-4′-thiocyano-disulfonate, was shown by Maddy[380] to react extensively with proteins exposed on the outside of bovine erythrocytes, but the amino phospholipids did not react significantly with the probe, suggesting these were located on the cytoplasmic surface of the membrane. This was essentially confirmed by Bretscher[381,382] using the impermeant reagent [$^{35}$S]-formylmethionyl sulphone methyl phosphate. Whitely and Berg[383] synthesized a novel imidoester, isothionyl acetimidate, which cannot penetrate the human erythrocyte membrane but has the same reactivity towards amino groups as ethyl acetimidate, which does penetrate the membrane. Using $^{14}$C and $^{3}$H-derivatives of the respective reagents, they showed that the cytoplasmic surface had ten times more reactive sites than the

external surface of the membrane, and nearly all these sites were found to be amino phospholipids. Furthermore, they reported that there was no drastic rearrangement of reactive groups on preparing isolated membrane preparations.

Additional evidence for lipid asymmetry has been obtained in the case of glycolipids of erythrocyte membranes which are located almost exclusively on the outer surface of the membrane.[384, 385] X-ray and neutron diffraction studies of myelin[341,386, 387] have also indicated some molecular asymmetry in this membrane. Casper and Kirschner[348] have suggested that asymmetry arises in the dispostion of the phospholipid and cholesterol components with an approximately equimolar ratio of cholesterol and polar lipid in the outer leaflet of myelin, but this ratio decreases to about 3:7 on the inner side of the membrane. London et al.[388] have suggested that differences in lipid specificities of the two myelin proteins (basic and Folch-Lees) support the idea of lipid and protein asymmetry in the myelin membrane. Cholesterol also appears to be distributed unequally between the outer and inner halves of the lipid bilayer of *Mycoplasma* and other cell membranes.[390] The asymmetry of phospholipids in the envelope of influenza virus has been examined, and the methods whereby this is achieved and maintained during viral budding has been reported.[391,392]

While analyses of asymmetric distribution in membranes have been directed towards establishing the location of the particular lipid classes, it should be emphasized that each of these components possess characteristic hydrocarbon chain residues. For example, the acidic phospholipids of the erythrocyte membrane tend to have comparatively unsaturated hydrocarbon chains. The choline phosphatides including sphingomyelin and phosphatidylcholine that are located predominently on the outer surface of the membrane possess realtively saturated hydrocarbon chains, which suggests that the fluidity characteristics of the two leaflets of the membrane may well be different. The biological significance of lipid asymmetry or asymmetry of fluidity characteristics is at present unknown.

### C. Protein Disposition in Biological Membranes

The asymmetry of membrane protein disposition in membranes was originally shown in stained thin sections but can also be inferred from freeze-fracture electron microscopy with respect to the distribution of membrane-associated particles in complementary fracture faces of membranes cleaved in a direction parallel to the surface. Although the nature of the intermolecular forces which determine the distribution of particles between the two membrane leaflets is not clear, the fact that asymmetry is observed implies that these forces are not the same on both sides of the membrane. Furthermore, the particle density of particular membrane fracture planes appear to be relatively constant, suggesting that the distribution is a permanent rather than a transient feature of the membrane. As we have indicated above, the membrane-associated particles are likely to represent interpolated protein, but it is not certain that they constitute intact proteins because there is some evidence that covalent bonds can be broken by the shearing forces generated during membrane cleavage. In addition to establishing the structural asymmetry of membrane proteins, techniques have been devised to demonstrate that functional asymmetry of certain membrane-bound enzymes and multienzyme complexes also exist. We will now examine some of these methods and review the type of information that has been obtained.

#### *1. Radiochemical and Fluorescent Labeling Studies*

Reagents that react with amino groups of phospholipids also react with similar groups on membrane proteins. Nearly all of these reagents, because of their size, charge, or other polar feature are not readily permeable to membranes yet are capable of reacting vigorously and, in many cases, specifically with proteins exposed on the

membrane surface. The labels are usually designed so that extremely small amounts can be detected by radioactive or fluorescence techniques. Some reagents have the added advantage of being reactive under relatively mild conditions of pH, temperature, and ionic strength so that membrane integrity is not unduly perturbed and cell lysis is prevented. Nevertheless, many labeling reagents have a finite permeability through membranes and, in so doing, react with proteins on the inner surface of the membrane as well as cytoplasmic proteins. The reaction time must therefore be carefully controlled so as to accentuate the reaction with outer membrane proteins while at the same time minimizing labeling of proteins inside the cell. A useful reaction index has been to compare the relative specific labeling of outer surface membrane proteins with typical cytoplasmic proteins; hemoglobin, for example, has been used as a convenient marker in erythrocyte labeling experiments. Bretschers' radioactive thiol reagent has been used to investigate the disposition of proteins of the human erythrocyte membrane.[32,393] The reagent reacts primarily with amino groups and possibly with hydroxyl groups as well. Labeled proteins of intact and ruptured membrane preparations were extracted and resolved by polyacrylamide gel electrophoresis and further characterized by amino acid fingerprinting techniques. A single protein was found to be labeled from both sides of the membrane, suggesting that it extends completely through the membrane and resides in a fixed orientation with respect to the inner and outer surfaces of the membrane. The diazonium salt of [$^{35}$S]-sulphanilic acid also reacts with protein amino groups,[394] but only low levels of labeling can be performed with confidence. When high concentrations are used, the resulting protein derivatives cause the membrane to become leaky to sodium and potassium ions, and the cells eventually lyse.[395,396] Tinberg et al.[397] and Schneider et al.[398] have used [$^{35}$S]-diazobenzene sulphonate to examine the disposition of proteins in intact mitochondria and preparations of inner mitochondrial membranes. A fluorescent probe, fluorescamine, has also been used to examine reactive groups on the inner and outer surface of the plasma membranes.[399]

A different procedure has been introduced by Rifkin et al.[400] involvng the formation of a Shiff's base between free amino groups of protein and pyridoxyl phosphate followed by reduction with $NaB^3H_4$. They argued that since pyridoxyl phosphate is impermeable to most membranes, the initial pyridoxylation and consequently the labeling reaction should be confined exclusively to proteins on the outside of the cell. This was tested on influenza virus which contains five membrane proteins, four of which are glycoproteins, exposed on the outer surface of the envelope, and the remaning protein is believed to be associated exclusively with the inner surface of the membrane. They were able to confirm that only glycoproteins exposed on the outside of the viral envelope were labeled, and all these could be removed by protease digestion of the intact virus particles. Probably the greatest disadvantage of the method is that protein amino groups vary in susceptibility to pyridoxylation so that it is difficult to compare the proportions of different membrane proteins.

A similar method for labeling sialic acid residues of membrane glycoproteins has been reported by Blumenfeld et al.[401] In this case the sugar is converted to an aldehyde by mild oxidation with periodic acid and then reduced to 3-deoxy-5-acetamidoheptulonic acid with $NaB^3H_4$ to introduce the radioactive label. Membrane proteins of the erythrocyte, other than sialoglycoprotein, were not significantly labeled in intact cells although various lipids including glycolipids, plasmalogens, and lipid peroxides were all extensively labeled. It was also reported that labeling efficiency was low in intact cells compared with isolated membrane preparatios, suggesting that perturbations in membrane structure may improve the conditions for oxidation-reduction reactions.

As we have noted already, the principal objection to the use of small molecular weight reagents is that they can and do penetrate the membrane slowly and react with

intracellular proteins. Tedious measurements of membrane permeability, or comparing the labeling patterns of intact cells with those of lysed preparations, do not always provide a satisfactory answer to this problem. One method used to overcome this difficulty is to employ high molecular weight-labeling reagents which are unquestionably too large to penetrate through the intact membrane. A popular procedure is to covalently link a small molecular weight reagent to a large sugar polymer. The advantages of carbohydrate polymers is that their size can be varied as required and in reactions where the label becomes covalently attached to the membrane, the sugar residues can be removed subsequently with glycosidases or left *in situ* to serve as specific antigenic sites. Lipid-soluble reagents have also been adapted for use in labeling studies. Schmidt-Ullrich et al.[402] describe a fluorometric method for labeling external proteins using dansyl chloride, a reagent commonly used for N-terminal analysis of proteins. The reagent is rapidly converted to an unreactive form in aqueous solution and must be protected by placing it in an apolar environment. This was achieved by incorporating dansyl chloride into aqueous dispersions of dipalmitoylphosphatidylcholine-cholesterol mixtures which then serve as the reagent vector. When the dye-complex was incubated with human or sheep erythrocytes for periods of up to 3 hr, membrane proteins were substantially labeled, but there were no apparent changes in the distribution of peptides on gel electrophoretograms, and the physical properties of the membrane as judged by osmotic fragility was unchanged. Moreover, hemoglobin did not react in intact cells, and only those peptides which label with other reagents were dansylated. Similar results were reported when isolated red cell membrane preparations were labeled by this method.

An alternative procedure using high molecular weight reagents is the use of enzymes to modify sites exposed on the membrane surface and then to label these sites in a subsequent reaction. Phillips and Morrison[403, 404] have developed a technique for iodination of exposed tyrosyl residues of membrane proteins using the enzyme lactoperoxidase (mol wt 78,000). The initial studies were performed on intact erythrocytes where it was found that only sialoglycoprotein and band 3 glycoprotein become iodonated in contrast to isolated membrane preparations when most of the membrane proteins were labeled. These studies have since been extended by combining limited proteolysis with lactoperoxidase catalyzed iodination of erythrocyte membrane proteins.[405] Reichstein and Blostein[406] adopted a novel approach to study protein disposition in the red cell membrane by using both radioactive isotopes of iodine. The inner surface of the erythrocyte membrane was labeled with iodine by trapping lactoperoxidase inside lysed membrane preparations and resealing them. After reaction with one iodine isotope the cells were washed to free them of excess iodine, and then proteins exposed on the outer surface of the washed membranes were labeled with the other isotope of iodine. Comparison of the radioactive profiles of peptides separated by gel electrophoresis confirmed previous observations that a peptide of apparent mol wt 90,000 was labeled from both sides of the membrane.

A consistent feature of lactoperoxidase labeling is that a large proportion of potential labeling sites on the erythrocyte membrane appear to be inaccessible to the enzyme. Proteins extracted from the membrane with butanol, for example, are much more reactive, and all fractions become labeled.[403] Moreover, quantitative estimates of the number of iodinated sites in intact membranes indicate that only about 2% of the putative reaction sites become iodinated even assuming that there is only one reactive tyrosyl residue per glycoprotein molecule and no di- or triiodotyrosyl derivatives are formed. A possible explanation for the inefficient labeling is that the carbohydrate coat on the outer surface of the membrane prevents the enzyme from reaching most of the potential labeling sites. This view is consistent with the finding that removal of 20% of the total sialoglycoprotein from the membrane surface with trypsin results in

a ten-fold increase in lactoperoxidase labeling; removal of further protein causes a corresponding decrease in iodination. According to the current hypothesis, partial removal of the carbohydrate coat exposes tyrosyl residues on other membrane proteins but, since protease also removes potentially reactive groups, the number of labeling sites decreases. If one makes the same assumptions as before, the number of potential sites that become labeled even under these conditions is still only about 25% so that many tyrosyl residues must still remain inaccessible to the enzyme.

Labeling with lactoperoxidase has been applied to plasma membranes of other cells including normal and neoplastic lymphocytes,[407,408] viruses, and human platelet membranes. Like the erythrocyte membrane, proteins are asymmetrically labeled and some components of each membrane appear to be accessible to iodination from both sides of the membrane. Salton et al.[411] have used the technique to locate membrane ATPase of *Micrococcus lysodeikticul* on the cytoplasmic surface of the membrane and the topographical arrangement of membrane proteins in the intact myelin sheath has been investigated by Poduslo and Braun.[412] Apart from plasma membranes, studies have also been undertaken with mitochondrial membranes,[413,414] and asymmetry of these membranes has been confirmed. Clarke[415] used a combination of iodination catalyzed by lactoperoxidase linked to Sepharose and labeling with the diazonium salt of sulphanilic acid to show that seven peptides were tightly bound to the outer surface of the inner mitochondrial membrane and were distinct from nine other peptides, accounting for between 50 and 60% of the total mitochondrial protein, loosely bound to the matrix surface of the membrane. Recent studies of membrane treated by the lactoperoxidase iodination method have indicated that lipids can also be iodinated.[416] Furthermore, lipid peroxidation due to addition of excessive amounts of hydrogen peroxide can be a serious problem leading to substantial changes in the properties of the membrane.[417] This can usually be prevented by the use of lipid antioxidants such as butylated hydroxytoluene and avoiding the use of large amounts of hydrogen peroxide. Care must also be exercised in identifying auto-labeled lactoperoxidase, which has occasionally been mistaken for a membrane protein.

Enzymic labeling procedures have been developed for glycoproteins; Gahmberg and Hakomori,[418] for example, have described an enzymic method based on a variation of the borohydride reduction procedure. The primary hydroxyl groups of galactose and N-acetyl galactosamine are oxidized with galactose oxidase (mol wt 75,000) and the products then reduced by $NaB^3H_4$. Glycoproteins of intact erythrocytes were highly labeled by this procedure, and no cell lysis was detected. Surprisingly, when isolated membrane preparations were reacted, an additional glycoprotein of mol wt 150,000 becomes labeled, suggesting that in intact cells this protein is unreactive because of its location on the cytoplasmic surface of the membrane. A more likely explanation, however, is that the glycoprotein becomes accessible to oxidation as a result of its rearrangement in the membrane during cell lysis.

### *2. Controlled Proteolysis*

When intact cells are treated with proteases, proteins exposed on the outer surface of the plasma membrane are selectively cleaved.[419] In most cases the digested membrane remains intact and impermeable to the enzymes despite the fact that considerable amounts of peptide may be released. This means that intracellular proteins, including those exposed on the cytoplasmic surface of the membrane, are protected from attack. Unfortunately, not all cell types behave in this way and many are rapidly lysed by tryptic digestion. A number of tissue culture cells and lymphocytes fall into this category; consequently, membrane protein disposition cannot be examined by this procedure. After protease treatment, membranes can be isolated and specific peptides identified by comparison with gel electrophoretic patterns of undigested membranes.

Controlled proteolysis can also be applied to membrane preparations in the form of resealed vesicles able to exclude enzymes, but precautions must be taken to ensure that all the membrane is oriented in the same way. Protease digestion of resealed membrane preparations has shown that, in general, relatively more protein can be released from the surface of these vesicles than from an equivalent surface area of plasma membrane of intact cells.

The type of peptides released obviously depends on the particular enzyme used. Many proteases have been used for this purpose including endopeptidases such as *Bacillus subtilis* protease, *Streptomyces* protease, papain, bromelin, and ficin all with nonspecific action as well as specific endopeptidases like trypsin, which hydrolyzes bonds adjacent to arginine or lysine and chymotrypsin, which attacks peptide linkages involving aromatic amino acids. The removal of terminal amino acid residues with carboxypeptidases A and B and leucine aminopeptidase from membrane proteins has also been investigated. Trypsin, chymotrypsin, and papain have all been used by Steck et al.[420] to investigate protein asymmetry in erythrocyte membranes. They monitored the rate and extent of digestion of membrane vesicles prepared with normal and inverted orientations by measuring the release of protons during the reaction. Judging from the amount and rate of proton generation, more proteins are susceptible to hydrolysis on right-side-out vesicles than on inverted vesicles. Furthermore, according to the polyacrylamide gel electrophoretograms, all the major membrane proteins, with one exception, including the sialoglycoproteins were vulnerable to attack in normally orientated vesicles as well as membrane ghost preparations whereas only one peptide and the sialoglycoproteins were accessible in inverted vesicles. It was shown that the peptide not degraded in right-side-out membrane was also protected from hydrolysis in inverted vesicles, indicating that it was located at an internal membrane site not inaccessible to proteases from either surface. One major disadvantage of the method is that protein may be removed from the membrane, or substantial rearrangements may take place during membrane isolation or when preparing membrane vesicles with the required orientation. This can be checked by forming derivatives of membrane proteins on the intact membrane before proteolysis thereby allowing the products to be identified as particular derivatives of chemically modified proteins, and their disposition may be compared with the products released from resealed membrane vesicle preparations.

Controlled proteolysis has also been employed to examine the interaction of individual membrane proteins with the bilayer structure. The rationale for these experiments is that those regions of the polypeptide chain residing in an aqueous environment will be susceptible to protease attack whereas those interpolated into the lipid bilayer will be protected. The analysis of various proteins by controlled proteolysis has shown that a large number of membrane-bound polypeptides exist as multidomain entities with regions located in the aqueous environment and others in the bilayer.[421] These include cytochrome $b_5$ of rat liver microsomes, a monomeric protein which is split into three fragments by proteolysis.[422,423] One fragment that is released to the aqueous phase is globular in structure and possesses the active site of the protein. Another fragment consists of a high proportion of hydrophobic amino acid residues and is required to anchor the protein in the membrane, detergent micelles, or phospholipid bilayers. The third component is small and has not yet been isolated or characterized, but its existence is inferred by the fact that neither the molecular weight nor the circular dichroism spectrum of the whole complex can be accounted for from the corresponding parameters of the two larger fragments in isolation. Other examples of multidomain proteins are NADH-cytochrome $b_5$ reductase responsible for the reduction of cytochrome $b_5$ of the microsomal electron transport chain,[424,425] erythrocyte MN sialoglycoprotein,[36,426-429] and Thy 1 antigens of thymocytes and brain.[430] Another group of pro-

teins can be recognized that split into fragments which remain associated with the membrane, suggesting that a portion of the polypeptide chain is exposed to protease enzymes, but much of the protein is interpolated into the hydrocarbon domain of the membrane. Examples of these proteins include $Ca^{2+}$-activated ATPase of sarcoplasmic reticulum,[431-433] rhodopsin of the retinal rod outer segment thylakoid membrane,[434] and band 3 glycoprotein of the human erythrocyte membrane.[435] In the last example, a water soluble fragment can be released from the cytoplasmic surface by trypsin and papain digestion, and this is believed to represent the binding site for glyceraldehyde-3-phosphate dehydrogenase on the membrane surface.

*3. Physical Studies*

As discussed in Section II.B.6, a variety of physical techniques have been applied to the study of the structure of membrane proteins. Detailed studies have been undertaken of the arrangement of bacteriorhodopsin within the purple membrane of *Halobacterium halobium* using electron diffraction methods.[74,75] The technique consists of taking electron micrographs at electron doses which are not completely destructive and compensating for the poor contrast by averaging over a large number of unit cells in an ordered arrangement of the specimen. The model of bacteriorhodopsin constructed from the electron diffraction data and viewed approximately parallel to the plan of the membrane is illustrated in Figure 12. About 80% of the polypeptide chain of bacteriorhodopsin is in an $\alpha$-helical structure with most of the chain embedded in the bacterial membrane. The helical portions are folded back and forth across the membrane to give seven discrete helical segments running approximately parallel to one another and perpendicular to the plane of the membrane. The lipid bilayer regions fill the spaces between the protein molecules.

*4. Functional Studies*

A considerable number of membrane-bound proteins express some form of enzyme activity and endow certain membranes with particular functional capabilities. These include the active transport proteins, cytochromes and components of electron transport chains, and synthetic and metabolic enzymes. It is possible to localize certain enzymes within the ultrastructure of thin sections by suitable modifications of standard histochemical techniques used in visible light microscopy.[436] Sections can be prepared with sufficient fixation to preserve morphological features without inactivating the enzyme, and the accumulation of reaction product can be observed by coupling it with a secondary capture reaction involving an electron-dense metal. Most hydrolase and redox reactions can be detected by the formation of organic polymers with osmiophilic properties at the reaction site. In many instances the procedure has enabled the location of enzymes at precise membrane surfaces. Cytohistochemical methods have been used by Seligman et al.,[437] for example, to demonstrate that cytochrome oxidase activity of the inner mitochondrial membrane culminates in the oxidation of cytochrome c on the outer surface of the membrane.

Functional sidedness of mitochondrial membranes with respect to solute transport has been demonstrated by Harris and Manger.[438] They examined respiration rates of substrates added either individually or in selected combinations to intact mitochondria. It was found that oxidation of $\beta$-hydroxybutyrate was additive in combination with various carboxylic acid-cycle intermediates, with the exception of succinate, where there was evidence of a competition presumably because succinate accumulation by mitochondria was inhibited by $\beta$-hydroxybutyrate. This and other evidence indicated that $\beta$-hydroxybutyrate was confined in a compartment separate from the other metabolites which competed against each other for transport into the mitochondria. The location and structural specificity of these transport proteins using similar methods to

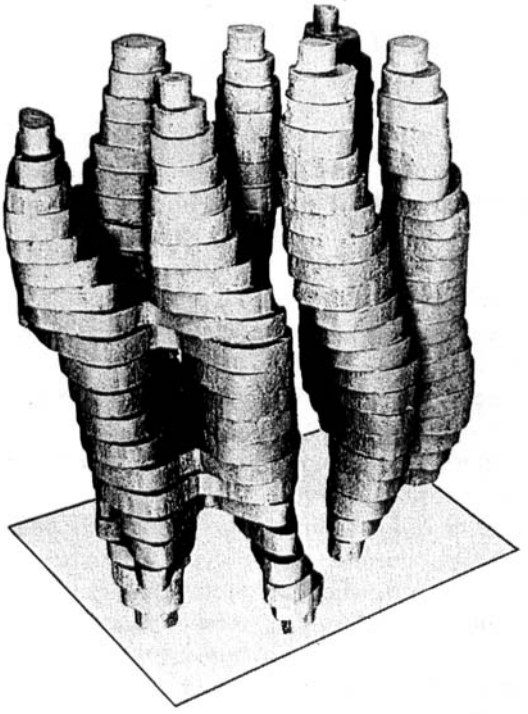


FIGURE 12. A model of a single intrinsic membrane protein, bacteriorhodopsin of the purple membrane of *Halobacterium halobium*. The model was constructed using electron diffraction methods and is viewed roughly parallel to the plane of the purple membrane. The protein is arranged in seven $\alpha$-helical segments which completely span the lipid bilayer membrane. The top and bottom portions of the model correspond to parts of the protein in contact with the aqueous medium, the remainder being in contact with the hydrocarbon region of the lipid. The most strongly tilted $\alpha$-helices are in the foreground. (From Henderson, R. and Unwin, P. N. T., *Nature (London)*, 257, 28, 1975.)

those described above as well as light-scattering techniques has been described by Chappel et al.[439] Now that satisfactory techniques have been devised to isolate and purify certain membrane components, the possibility of raising specific antibodies against these proteins has been explored. Racker et al.[440] have prepared anti-$F_1$ antibodies which were found to interact with inverted inner mitochondrial membrane vesicles but not with intact mitochondria. In contrast, antibody against cytochrome c can inhibit succinoxidase only when added to intact mitochondria, but antibody against cytochrome oxidase can interact from both sides of the mitochondria. In other studies,[441] site-specific antibodies have made it possible to postulate specific loci on cytochrome c as sites of interaction with cytochrome $c_1$ in the mitochondrial membrane.

Functional sidedness is also implied in the case of the adenylcyclase complex located in the plasma membrane of many mammalian and other cells. Hormones stimulate the active site of the enzyme allosterically by binding to a protease-sensitive receptor site located on the outside surface of the membrane. The catalytic site, on the other hand, is believed to be located on the cytoplasmic surface of the membrane because the substrate must be supplied from within the cell and the products are released to the cytoplasm. Thus substrate added outside the cell is not converted into cyclic AMP

unless the membrane is rendered permeable to ATP. Likewise, several membrane transport proteins[442] including ($Na^+$-$K^+$)-ATPase have been shown to exhibit functional asymmetry. Whittam and Ager[443] working with erythrocyte membranes, and Baker[444] with crab nerve, showed that ATP and $Na^+$ interact with sites on the enzyme accessible only from the cytoplasmic surface of the membrane whereas $K^+$ interacts with an external site. The ADP and inorganic phosphate resulting from the potassium-stimulated phosphatase activity are both released into the cytoplasm despite the external location of the $K^+$ binding site.[445]

*5. Lateral Asymmetry of Membrane Protein Disposition*

Many physiological events rely on contact between different membranes of the cell, or different regions of the same membrane. These contacts may be stable and constitute distinct morphological features such as myelin, retinal rod outer segment membranes, and chloroplast thylakoids, or they may be unstable an lead to fusion of the two membranes. The latter includes such processes as endocytosis and exocytosis, in addition to pathological mechanisms like phagocytosis. Since many specific membrane contacts are restricted to certain regions of the membrane, it is often assumed that these areas become specialized in some way to facilitate interaction. Glycoprotein must assemble into patches on the surface of the plasma membrane before cells will agglutinate.[446] Fusion of spermatozoa takes place when contact between the two gametes is established through the acrosomal region of the sperm. Accordingly, the outer actosomal membrane possesses sites capable of recognizing the ovum in addition to components needed to penetrate the ovum once the outer acrosomal membrane has been shed. Thus, concanavalin A binding to sites that are located exclusively on the plasma membrane overlying the acrosome[447] causes agglutination of the spermatozoa and prevents fertilization, suggesting that a redistribution of sites may be involved in interaction with the ovum.[448] The male H-Y antigen of mouse spermatozoa is restricted to the acrosomal membrane[449] since no antigenic sites can be detected on the plasma membrane overlying other regions of the cell. Localization of other membrane receptor sites on the plasma membrane have been reported in nerve tissue. Lectin binding sites specific for concanavalin A and ricin[450] are located almost entirely in the synaptic membrane. Acetylcholine receptors of the neuromuscular junction are also confined to the area of the end plate in innervated striated muscle and appear to remain fixed even after denervation.[451]

A redistribution of membrane components appears to be required in the formation of stable junction complexes between cells. These junctional complexes include desmosomes, zona and macula occludens, and gap junctions and are regarded as differentiated forms of the plasma membrane rather than as separate organelles.[452] The complex appearance of zona occludens when examined by freeze-fracture electron microscopy suggests that a considerable rearrangement of components is required to form the complex,[453] and it is likely that specialized proteins are located in this region. Evidence that this is the case with gap junctions is more convincing. The junction tissues including cardiac and smooth muscle, epithelial and connective tissues, and at electrical synapses in the nervous system. Microelectrode studies have demonstrated that gap junctions electrically couple cells, and other mutant studies have shown that interacting cells are also metabolically coupled.[454] Channels or pores up to 1 nm in diameter are believed to exist in the region of these junctions.[455]

The requirement for specialized membrane proteins in the formation of gap junctions has been obtained from an interesting series of experiments described by Azarnia et al.[456] in which the ability of cells to form gap junctions appears to be genetically controlled. They fused a human cell line, in which gap junctions are the only discernible form of cell contact, with a mouse cell line incapable of establishing any kind of

cell junction. All the heterokaryons formed electrically coupled gap junctions, and an amost complete set of parent chromosomes. Some clones derived from among the segregants again lost the ability to form gap junctions, and these revertants were invariably deficient in human chromosomes. It was concluded that human chromosomes supply a genetic factor which corrected a deficiency in the mouse cell line — namely, factors responsible for gap junction formation — in a manner suggesting that the mouse cells were genetically recessive for this characteristic. It is possible that genetic control is exercised by directing the synthesis of specific membrane proteins that are required in the formation of the junction complex.

In contrast to plasma membranes in general, gap junctions are notably resistant to disruption with certain detergents. This indicates that the type or particular arrangement of membrane components in the region of the junction may be different. From an operational viewpoint, this property has provided a convenient method whereby gap junctions can be isolated and analyzed.[457, 458] Mouse liver gap junctions isolated by detergent treatment appear to have a higher proportion of neutral lipid and a distinctly different phospholipid composition from other regions of the plasma membrane. There is generally more lipid relative to protein and a single peptide of about 20,000 mol wt predominates. Comparison of the metabolic turnover of these gap junction proteins with the turnover rate of proteins located in other regions of the plasma membrane has indicated that the junction proteins are extremely metabolically stable an may be related to the fact that they occupy a relatively fixed position in the membrane.[459] Some caution should be exercised in accepting such results as anything other than preliminary at this stage, especially in view of the fact that detergents may selectively remove some membrane components from the junction complex without necessarily altering the morphology of the membranes. Nevertheless, if there are any true differences in membrane composition this must reflect to some extent the need to preserve the integrity of membranes when they come into close contact during formation of the junction complex. By analogy, one might expect the composition of membranes in regions involved in unstable interactions and fusion to be altered as well, but, as yet, there is no evidence available to substantiate this view.

### D. Motional Freedom of Membrane Proteins

It is clear from our discussion in the last section that proteins are arranged asymmetrically in various membranes. The method by which proteins are interpolated into the structure and the physicochemical properties of the polypeptides is largely responsible for maintaining this asymmetry. This means that the orientation of membrane proteins with respect to the plane of the lipid bilayer is relatively fixed for each particular protein. This does not imply that all proteins are fixed rigidly in the membrane since it has been clearly demonstrated that certain proteins can rotate about an axis perpendicular to the plane of the membrane and to diffuse laterally through the lipid bilayer. Indeed, a lateral asymmetry of the disposition of some proteins in membranes can be induced by a number of mechanisms, and these probably constitute important factors in modulating cellular function and behavior. There are several reviews dealing with rotational and lateral diffusion of membrane proteins,[460-462] and we shall confine our discussion to the principal evidence upon which the motion of protein constituents of biological membranes is based.

#### *1. Rotational Motion of Membrane Proteins*

Evidence that proteins rotate about an axis perpendicular to the plane of the membrane was first obtained from studies of the visual pigment, rhodopsin. The highly regular parallel alignment of thylakoid membranes in retinal rod outer segments has

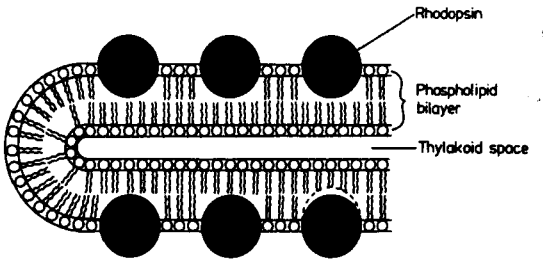



FIGURE 13. A molecular model of the frog retinal rod outer segment membrane. The membranes form closed vesicles or thylakoids which are stacked in layers oriented perpendicular to the axis of the outer segment of the rod cells. The rhodopsin molecules shown as dark circles are oriented on the outer surface of the membrane and extend into the hydrocarbon region to an extent dependent on the state of bleaching of the associated retinal. The dimensions of the respective membrane components are given in the text.

enabled the performance of detailed studies of the arrangement of the major protein in these membranes.[463] A model of the arrangement of rhodopsin in the membrane is presented in Figure 13, which is based on these studies and includes information obtained from spectroscopy, electron microscopy, X-ray diffraction analysis, and osmotic measurements. The rhodopsin molecule is a glycoprotein of molecular weight of about 40,000 and roughly spherical in shape with a diameter of about 4.5 nm. The spheres are submerged 1.4 or 2.1 m, within the lipid (hydrocarbon) region depending on the light conditions. The total thickness of the membrane varies accordingly between 7.0 and 6.3 nm and is a composite of the rhodopsin component protruding into the aqueous phase and a phospholipid bilayer of 4.7 nm, of which 3.7 nm represents the dimension of hydrocarbon region. Spectroscopic studies have shown that the retinal rod is strongly dichroic in that light polarized perpendicular to the long axis of the rod is strongly absorbed compared with light that the chromophore of rhodopsin, retinal, is preferentially orientated parallel to the plane of the thylakoid membrane. Initial studies of partial bleaching with short flashes of polarized light lacked sufficient time resolution to demonstrate dichroism in the plane of the membrane, and it was concluded that rotation of the molecule about an axis normal to the membrane had a relaxation time faster than 100 ns.[464] The transient nature of dichroism was obtained using a fluorescent derivative of rhodopsin, N-retinyl opsin, where it was shown that dichroism lasts only as long as the molecule takes to rotate about its axis for which a lower limit of 26 ps for the rotational relaxation time was determined.[465] If rotation is prevented by fixing the molecule in the membrane with bifunctional cross-linking reagents like glutardialdehyde, then strong dichroism can be demonstrated.[466] The rotational relaxation time of rhodopsin has been accurately measured by Cone[467] using a flash photolysis apparatus capable of resolving events in the microsecond time range. In experiments with frog retina, intermediate compounds in the bleaching sequence of the rhodopsin molecule were produced by exposing the retina to extremely short (5 ns) flashes of light and then the decay of the ensuing dichroism with perpendicular and parallel polarized light was measured. By selecting appropriate wavelengths to distinguish bleaching products from residual unchanged rhodopsin, it was possible to measure the rotational relaxation time of the rhodopsin molecule in the membrane. The value obtained was in the order of 20 $\mu$s at 20°C. From a knowledge of the rate of rotation of rhodopsin, the viscosity of the surrounding lipid matrix could be calculated, and this was in reasonable agreement with values obtained for the hydrocarbon region of fluid membranes.

Similar studies have been reported for bacteriorhodopsin located in the purple membrane of *Halobacterium halobium* in which a transient spectroscopic chromophore centered at 410 nm was used to investigate rotational diffusion.[468] The absorbance has a lifetime of about 10 ms and was found to be strongly dichroic following flash illumination with plane polarized light, but in contrast to rhodopsin this did not decay rapidly. The rotational relaxation time was found to be at least $10^3$ times slower than rhodopsin in the retinal rod outer segment disc membrane consistent with the crystal-like hexagonal packing of bacteriorhodopsin in purple membranes inferred from X-ray analysis[469] and unlike the liquid-like array of rhodopsin in the thylakoid membrane.[470] Examination of the photolytic dissociation of cytochrome $a_3$-CO complex in the inner mitochondrial membrane[471] and photoinduced dichroism of chlorophyll $a_1$ in chloroplast membranes[472] has also suggested that these proteins do not rotate rapidly about an axis perpendicular to the membrane.

Fluorescence techniques such as those designed to measure rotational diffusion of probes in lipid bilayers[473-476] and membranes[477,478] and of proteins in free solution[479] have been applied to study protein rotation in membranes.[480, 481] The fluorescence polarization of analino naphthalene sulphonate and dansyl chloride bound to electroplax membrane fragments, for example, have been resolved into two components: a rapid but partial decay, believed to be rotation of the probe at its binding site; the residual decay due to rotation of the protein which set a lower limit for rotational relaxation time of 0.7 $\mu$s.[482] Fluorescent-labeled lectins bound noncovalently to the surface of normal and transformed lymphocytes, and fibroblasts have been used to investigate rotation of membrane proteins but with limited success.[483,484] Tryptophan fluorescence has been explored as an intrinsic probe to measure rotational kinetics of other membrane proteins, and external probes such as eosin have been used successfully in model systems and erythrocyte membranes to study the rotation of proteins which have no natural chromophores.[461] It should be noted that there is some ambiguity in interpreting the data obtained from these fluorescence methods because the spectroscopic features are generally considered to arise from a rigid molecule in which the chromophore is inflexibly integrated. Care must therefore be taken to ensure that no component of the spectral parameters are due to rotation about its binding site to the membrane protein independent of rotation of the receptor protein in the membrane. In general, however, these studies indicate that rotational relaxation times for different membrane proteins vary considerably and probably reflect differences in the molecular environment surrounding each particular protein.

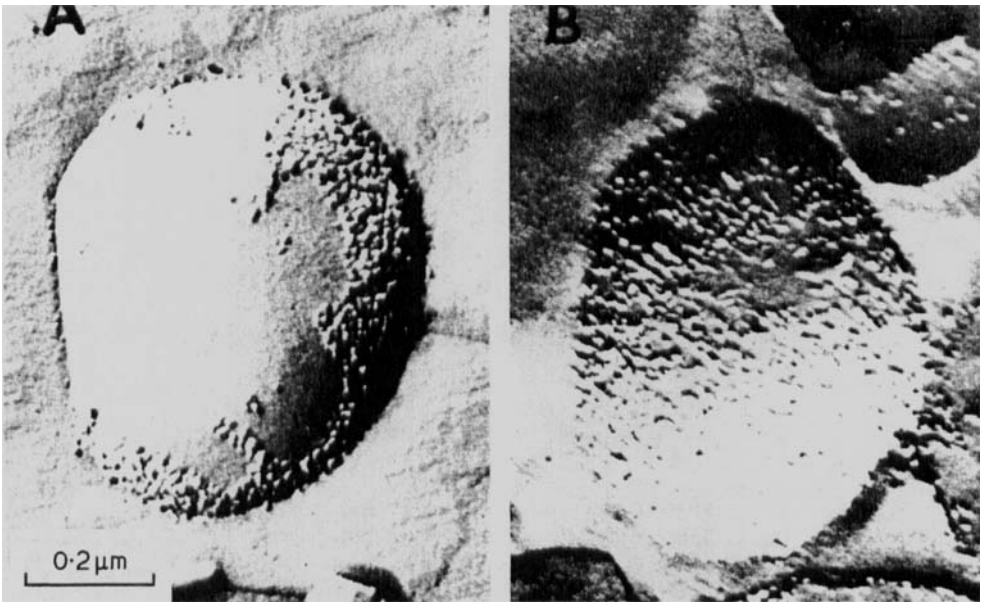
### *2. Protein Aggregation*

Changes in the distribution of protein in the plane of the membrane was first noted by Pinto da Silva[485] when he examined freeze-cleaved erythrocyte membrane preparations that had been quenched from acidic media. Membrane-associated particles are distributed randomly on complementary inner fracture faces of these membranes at pH 7.5 or pH 9.5, but they aggregate when the pH is reduced to 5.5 or less. Adjusting the pH again to 7.4 restores the original particle distribution so that the process is freely reversible. Changes in particle distribution in the hydrophobic region of membranes appears to be controlled largely by interactions taking place in the aqueous phase because increasing the electrolyte concentration prevents particle aggregation at low pH. The reason why particles aggregate at low pH is not yet clear, but it has been suggested that the situation may be analogous to the precipitation of soluble proteins near their isoelectric point. Cross-linking with glutardialdehyde or formaldehyde also prevents particle aggregation, and this treatment is believed to anchor proteins more securely in the membrane and prevent their lateral movement in the plane of the membrane.

The aggregation of membrane-associated particles in erythrocyte membranes at low pH also appears to be correlated with changes in the disposition of specific protein receptors exposed on the outer surface of the membrane. Colloidal iron hydroxide and ferritin derivatives of antibodies directed against specific membrane proteins have been used as electron microscope markers to observe changes in the distribution of surface receptor sites on the erythrocyte membrane.[486,487] Colloidal iron hydroxide, which binds preferentially to N-acetyl-neuraminic acid residues most of which are attached to the sialoglycoprotein of the red cell membrane, was found to be reversibly aggregated at low pH in the same way as membrane-associated particles of the inner membrane fracture face. Moreover, antibody prepared against spectrin, an extrinsic protein attached to the cytoplasmic surface of the erythrocyte membrane, not only aggregated this protein but also induced an aggregation of colloidal iron hydroxide bound simultaneously to the sialoglycoprotein on the outer surface of the membrane.[487] The nature of the coupling between spectrin and sialoglycoprotein is not known, but since spectrin is an extrinsic membrane protein presumably absorbed to the cytoplasmic membrane surface predominatly by electrostatic interations, the connection between the two proteins is likely to be fairly nonspecific. In any event, the results of these experiments suggest a possible mechanism whereby the distribution of cell-surface components can be altered by intracellular interactions. Interactions between proteins on opposite sides of the membrane is thought to be involved in the maturation and budding of enveloped virus from host cells. The envelope, although derived from the host plasma membrane, does not contain proteins normally associated with this membrane, and the mechanism that sequesters viral-specific proteins into the region destined to form the envelope is though to be the alignment of another, viral-specific protein on the cytoplasmic surface of the host membrane.[488]

Freeze-fracture techniques have been used in conjunction with spin-label probes to examine membrane-associated particle distribution of chloroplast membranes.[489] Particle distribution in these membranes appear to be random in adapted chloroplasts, but they become aggregated following illumination. A marked change in the partition of a spin-probe between the aqueous phase and the hydrocarbon region of the membrane correlated with an equivalent increase in temperature of 2°C. In other experiments, temperature-dependent, membrane-associated particle aggregation has been observed in various microorganisms including the alveolar and other membranes of *Tetrahymena Pyriformis*[490-494] and in plasma membranes of *E. coli*[495] and *Acholeplasma laidlawii.*[495-497] Protein-induced aggregation has also been reported in mitochondrial membranes[498] and lipid-reconstituted $Ca^{2+}$-activated ATPase of sarcoplasmic reticulum[499] but not in plasma membranes of lymphoid cells,[500] possibly due to constraints on the free diffusion of proteins in this membrane.[501] Proteins of some bacterial membranes also remain dispersed even when the membranes are cooled to below the lipid phase transition temperature.[502] Erythrocyte glycophorin also appears to be dispersed both above and below the phase transition temperature.[503] Some interesting studies of reconstituted membranes containing rhodopsin have shown that dark-adapted rhodopsin is aggregated below the lipid transition temperature but is dispersed randomly at higher temperatures. When the chromophore is bleached, however, the rhodopsin molecules remain dispersed at all temperatures.[504]

The relationship between membrane-associated particle aggregation and lipid crystallization has been demonstrated convincingly in the microorganism *Mycoplasma mycoides* var. *capri*[505] and illustrated in Figure 14. This shows freeze-fracture replicas of a strain of the organism devoid of cholesterol (Figure 14A) in which two regions can be observed, one with a high density of particles, the other smooth and containing no particles. Similar replicas of the parent strain which contains cholesterol (Figure 14B)




A B

FIGURE 14. The relationship between membrane associated particle aggregation and lipid crystallization in *Mycoplasma mycoides* var. *capri*. Cells from the cholesterol rich parent strain (b) and cells from an adapted strain in which the cholesterol content of the membranes was low (a) were suspended in 20% glycerol and equilibrated at 4°C prior to thermal quenching. Freeze-fracture replicas of the convex fracture faces of the respective cells are shown in which extensive smooth regions appear in the membranes of the adapted strain (a), which are believed to represent domains of crystalline lipid. No phase separations are observed in membranes of the cholesterol-rich parent strain (b), and the particles which are likely to consist of intrinsic membrane proteins remain randomly distributed in the plane of the membrane. (Electron micrographs by Rottem et al.[505]).

show that the proteins remain dispersed more or less randomly throughout the plane of the membrane.

Protein aggregation within the fluid lipid bilayer triggered by mechanisms other than lipid crystallization has been related to various cell processes including pinocytosis,[506] the stage of the cell growth cycle,[507] and to membrane interactions[508,509] and fusion.[510] The factors which determine the state of aggregation of protein within a lipid membrane above and below the lipid transition temperature remain, however, a subject of speculation.

We have devised simple model experiments to simulate the events that take place when the lipid chains of a randomly ordered array of lipid and polypeptide chains crystallize. This model envisages the spontaneous lipid protein distribution in the fluid state (above the phase transition temperature of the lipid) upon cooling to below the phase transition temperature. At very high protein content the lipid will be unable to freeze and crystallize, whereas in the intermediate range of protein concentrations, the regions of the membrane most affected by the crystallizing lipid will be those which spontaneously possess very few protein-protein or protein-lipid-protein contacts. The crystallizing lipid will exclude the protein and at least part of the noncrystalline lipid surrounding the protein into regions in which the protein concentration is relatively high. The extent to which these protein-rich areas now run together will depend upon the rate at which the system is cooled. The various processes involved in the lipid crystallization and protein aggregation or protein-lipid clustering are illustrated sche-

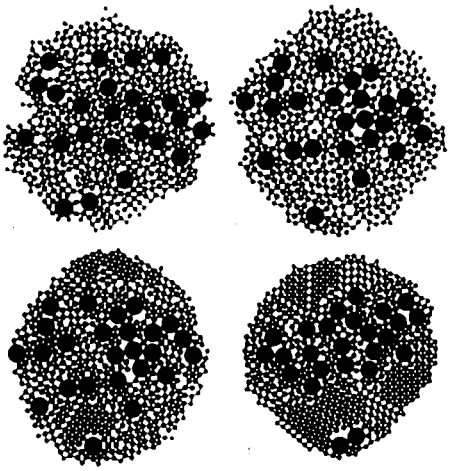


FIGURE 15. Schematic diagram showing how lipid crystallization can lead to a protein aggregation. (a) Random distribution of lipid and protein; (b) more extensive areas of lipid nucleate-producing domains of crystalline lipid intersected by packing faults seen in (c) the crystallization of certain areas of lipid forces protein molecules to aggregate; (d) equilibrium low temperature distribution of lipid and protein.



matically in Figure 15. This shows an initially random distribution of lipids and proteins (Figure 15A) in which areas of lipid nucleation appear (Figure 15B) producing domains of crystalline lipid intersected by packing faults. As crystallization of the lipid proceeds, protein molecules are forced to aggregate (Figure 15C) achieving an equilibrium distribution at low temperatures depicted in Figure 15D.

To extrapolate these observations to a natural membrane as distinct from a reconstituted or model membrane, we can expect a range of lipid classes and lipid acyl chains. When such membranes are cooled slowly, the higher melting point lipid will tend to crystallize out first. Depending upon the relative amount of intrinsic membrane protein, the crystallizing lipid will exclude the proteins into regions of lower melting point lipids producing a localized increase in protein-to-lipid ratio thus giving rise to protein aggregates or protein-lipid clusters. The spontaneous protein aggregates which are observed in the fluid or disordered structure of cell membranes, shown schematically in Figure 15A, may play an important role in certain membrane functions, including transport processes. It may be expected that, where membrane functions are influenced by protein dispositions, such spontaneous protein aggregates would be especially prevalent in membranes containing a relatively high protein-to-lipid content or where, as a result of a trigger mechanism, the local concentration of protein is increased.

### *3. Ligand-Induced Protein Rearrangements*

The aggregation of a number of cell-surface components has been adduced from studies of the binding of lectin[511-516] and antibody derivatives[517-525] directed against antigen locations on the outer surface of the plasma membrane of cells. The aggregation of receptor sites or antigens does not appear to require metabolic energy but does depend on the multivalency of the lectin or antibody, and the rate of aggregation is temperature-dependent. The aggregation of lectin binding sites on the erythrocyte sur-

face is associated with an aggregation of membrane-associated particles in the hydrophobic region of the membrane, but it is not known whether this connection extends to spectrin or other proteins located on the cytoplasmic surface. (c.f. Reference 488). A number of important cellular reactions such as cell contact and agglutination are believed to depend on the distribution of glycoprotein receptors on the cell surface. Many tissue culture cells, for instance, cease growing when they reach confluency and will agglutinate and stop dividing when treated with low concentrations of lectin such as concanavalin A. Certain variant cell lines, however, have been isolated which do not agglutinate and grow normally in the presence of relatively high concentrations of the lectin.[516] The variant strains usually display the same number of concanavalin A binding sites to which lectin appears to bind with equal affinity, but, unlike the parent strains, membrane-associated particles of the inner membrane fracture face do not aggregate, suggesting that particle aggregation or lectin binding site distribution is an important factor in cell agglutinability.

An interesting effect on the distribution of lectin binding sites is observed when the cell surface is digested briefly with trypsin. Tryptic digestion causes normal cells to agglutinate and relieves density-dependent inhibition of growth in confluent monolayers of cells in tissue culture. Similarly, mild proteolysis also agglutinates cells from variant lines which are not normally sensitive to lectin and, in the presence of lectin, membrane-associated particles of these cells become aggregated. The action of trypsin on normal culture cells has been found to depend to some extent on temperature. Rosenblith et al.,[515] working with mouse fibroblasts, reported that concanavalin A binding sites were randomly distributed over the cell surface at 4°C or 37°C. These sites remain dispersed after trypsinization if the cells are kept cold but aggregate if the temperature is raised to 37°C. This implies that fluidity of membrane lipids is necessary for proteins to move laterally in the membrane and, furthermore, that removal of certain proteins from the membrane surface permits a multipoint attachment of the binding sites to lectin. Studies by Gunther et al.[526] have revealed that lectin binding sites aggregate because of the multivalent character of the agglutins. Using the agglutination of cells as a measure of the biological activity of concanavalin A, they showed that the native protein of valence 4 is many times more potent than a divalent derivative of the lectin even though the derivative appears to bind with equal affinity to the same membrane receptor sites. Other effects of concanavalin A, such as the ability to prevent the movement of immunoglobulin receptor sites over the lymphocyte surface, are also lost when the valence is reduced, suggesting that the multipoint attachment of lectin to a group of binding sites is the primary action of those proteins.

In general, ligand-induced protein aggregation involves the attachment of the lectin or antibody to the receptor protein which, through random collisions with other receptor molecules, becomes aggregated by cross-linking to the ligand into patches on the membrane surface. The random diffusion of proteins on the surface of cells has also been confirmed by other types of experiments. Fry and Edidin,[527] for example, examined the redistribution of histocompatibility antigens of a heterokaryon formed from the fusion of mouse (H-2 antigen) and human (HLA antigen) cells by an indirect fluorescent antibody technique. With 5 min of fusion the heterokaryon bore two separate populations of surface antigens, which became progressively mixed until, after about 40 min incubation at 37°C, total mixing was observed in 90% of the cells. A lateral diffusion constant of the order of $10^{-6}$ $m^2s^{-1}$ could be derived from the rate at which the histocompatibility antigens mixed. Although apparently independent of protein synthesis or metabolic energy, the process was markedly temperature-sensitive. Thus the rate of intermixing decreases with temperature in the range 37°C to 21°C but surprisingly increases in the range 21° to 15°C before again decreasing.[528] The behavior at intermediate temperatures may be a consequence of phase separation of the mem-

brane lipids.[529] Lateral diffusion rates of between 1 and 3 × $10^{-5}m^2s^{-1}$ have been obtained using similar antibody techniques for an antigen located on the surface of a muscle fiber in tissue culture.[530] A different procedure has been used to determine the lateral diffusion of rhodopsin in the thylakoid membrane. The method involves bleaching the molecules in one half of a single rod outer segment with a flash of light and following the subsequent intermixing of molecules (bleached and unbleached) in the whole membrane.[531,532] Complete randomization of molecules was observed in less than 1 min providing diffusion constants for the process of between 3.5 and 5.5 × $10^{-5}m^2s^{-1}$. Similar techniques using fluorescent-labeled proteins of erythrocyte ghost membrane preparations indicated that the labeled proteins of these membranes do not diffuse readily in the plane of the membrane,[533] a feature consistent with spin-label data.[534] Recently Schlessinger et al.[535] have reported the use of a fluorescence correlation spectroscopic method for measuring diffusion coefficients greater than $10^{-5}m^2s^{-1}$ and a fluorescence photobleaching recovery procedure to measure diffusion coefficients less than $10^{-5}m^2sec^{-1}$ to examine the lateral mobility of concanavalin A receptors on the surface of cultured myoblasts. Both methods were able to discriminate between diffusion and uniform-driven transport processes as well as between mobile and immobile fluorophore populations. They observed diffusion coefficients of between 0.8 and 3 × $10^{-7}m^2s^{-1}$ for the receptor complex which was two orders of magnitude slower than the mobility of a fluorescent lipid probe, suggesting that the receptor sites were aggregated by the lectin.

The movement of selected surface receptor sites around the periphery of a number of cell types, including lymphocytes, has now been reported. Thus, in unperturbed lymphocytes, antibody located on the surface of the plasma membrane is distributed randomly over the cell surface. If multivalent antibodies directed against these surface immunoglobulins are added to a suspension of cells, the cross-linked antigen-antibody complex migrates to one pole of the cell where it accumulates in the form of a cap (see Reference 517). The capping phenomenon can be observed directly if fluorescent anti-immunoglobulin is used or indirectly under the electron microscope with ferritin-conjugated anti-immunoglobulin.[536] The ferritin-conjugated antibody method has shown that capping is preceded by the formation of dense aggregates or patches of antibody bound to immunoglobulin receptors on the membrane surface. It has been suggested by de Petris and Raff[525] that cap formation in lymphocytes results from membrane flow analogous to the mechanism of cellular movements, and they cite the fact that cap formation is accompanied by morphological changes in the lymphocyte such as elongation and uropod formation, features which are usually associated with motile cells, to support their contention. At temperatures above 20°C, immunoglobulin molecules are distributed evenly over the lymphocyte surface and are thought to be maintained as independent antibody receptor sites by thermal agitation. Multivalent antibodies or lectins (in case of glycoproteins) effectively cross-link the surface receptors when they come into close proximity, and the complexed units then associate into patches located at several points on the cell surface (see Figure 16). These events do not require metabolic energy but are restricted at low temperature when random collisions between binding sites are presumably less frequent. Thermal agitation alone, however, cannot explain the direction of patches into a polar cap because the process is extremely rapid and is completed in less than 2 min at 37°C. Moreover, directional movement of patches to a particular region of the plasma membrane and the metabolic dependence of cap formation favor a more specific mechanism. A plausible theory to account for cap formation is that patches constitute regions of restricted fluidity in the membrane and may be anchored to contractile elements in the cytoplasm. Stabilization of the patch permits other, more fluid membrane components to flow directionally away from the region in which the cap is formed.

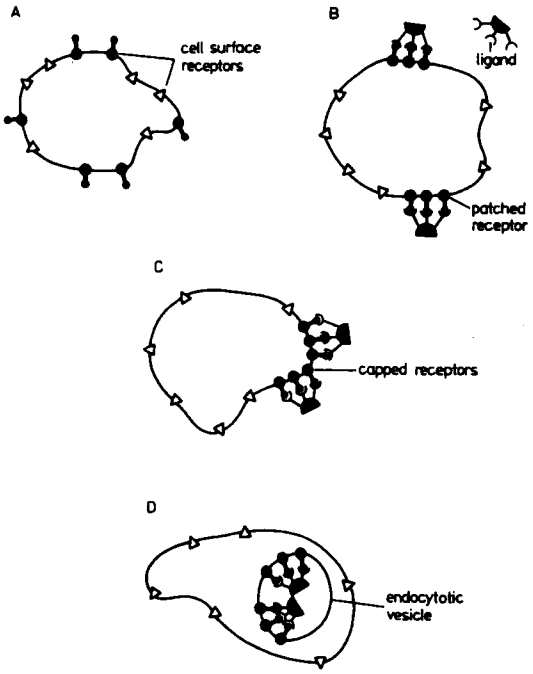



FIGURE 16. Schematic representation of the process of patching and capping of cell surface receptors induced by multivalent ligands. (A) Two different plasma membrane receptors ⌇ and △ are distributed randomly over the surface of the cell. (B) Ligands binding to one of the receptors, ⌇ , causes them to aggregate into patches located at separate sites on the cell surface. (C) The patches migrate laterally in the membrane around the cell periphery forming a cap at one pole of the cell. The other antigen, △, remains randomly distributed. (D) The membrane components associated with the respective ligands in the cap region may be internalized by a process of endocytosis.

The fate of selectively capped antigens appears to differ depending on the origin of the particular lymphocyte. In some cases of the antigen-antibody complex is taken into the cell by endocytosis and can be observed on the inner surface of intracellular vesicles, but in other lymphocytes the capped complexes are rarely internalized. This phenomena has been investigated by Stackpole et al.[537] who compared the migration of selectively capped antigens on mouse lymphocytes derived from either the thymus or spleen. Thymus-derived lymphocytes or T-cells are characterized by the presence of a specific antigen, TL, when obtained from the thymus and can be distinguished from B-cell lymphocytes, when recovered as a mixed T- and B- cell population from the spleen, by the presence of another surface antigen, Thy 1. B-cells, on the other hand, are the only lymphocytes from spleen to possess immunoglobulin receptor sites. Each type of lymphocyte participates in different immune reactions, but both have the same histocompatibility antigens (H-2) and lectin binding sites. The capping site of these antigens and receptors when the cells are exposed to appropriate antigens are presented in Table 18. It can be seen that immunoglobulin, Thy 1 antigen, and concanavalin A binding sites are capped over the Golgi pole of spleen lymphocytes, and they are almost completely removed from the cell surface by endocytosis. In contrast, Thy, TL, and histocompatibility antigens of thymocytes are capped predominantly at a pole opposite to that containing the Golgi apparatus and are only occasionally observed inside the

TABLE 18

**The Site of Cap Formation and Extent of Endocytosis of Surface Receptors on Mouse Lymphocytes**

| Lymphocyte | Receptor | Number of caps: Over Golgi | Number of caps: Opposite Golgi | Number of caps: Intermediate | Endocytosis |
|---|---|---|---|---|---|
| Spleen (mixed T- and B-cells) | Immunoglobulin | 28 | 0 | 2 | + + + + |
| | Concanavalin A | 10 | 2 | 0 | + + + + |
| | Thy 1 alloantigen | 12 | 2 | 0 | + + + |
| | H-2 | 10 | 11 | 8 | + + |
| Thymus | TL alloantigen | 2 | 18 | 5 | — |
| (T-cells) | Thy 1 alloantigen | 1 | 9 | 2 | ± |
| | H-2 | 3 | 12 | 5 | ± |

Data from Stackpole, C. W., Jacobson, J. B., and Lardis, M. P., *Nature (London)*, 248, 232, 1974.

cell. In other experiments, Stackpole et al.[537] were able to show that metabolic energy is required for capping irrespective of where the cap is located. Furthermore, cytochalisin B, an agent which disrupts cytoplasmic microfilaments, prevents cap formation of antigens directed towards the pole overlying the Golgi complex but has no effect on capping in thymocytes where the antigen-antibody complex migrates to the opposite pole of the cell. The role of microfilaments in directing cap formation and the significance of the site at which the cap forms in particular lymphocytes remains to be determined.

A selection process also appears to operate when areas of the plasma membrane become internalized during endocytosis or phagocytosis. When alveolar macrophages or polymorphonuclear leucocytes are allowed to ingest inert latex particles or oil droplets, more than half of the original plasma membrane may be taken up into the cell. Analyses of the remaining plasma membrane have shown that certain components are left on the cell surface while others are removed roughly in proportion to the amount of membrane internalized. Tsan and Berlin,[538] for example, measured the activities of transport systems for lysine, adenosine, and adenine in cells before and after phagocytosis of latex particles and found no decrease in the number of transport sites remaining in the surface membrane. The possibility that new transport sites had been inserted during phagocytosis was discounted in separate experiments where the transport sites were blocked with noncompetitive inhibitors before phagocytosis: the transport processes were apparently still blocked after latex particles had been ingested. It must be conceded that transport sites may be localized in certain areas of the plasma membrane not taken up during phagocytosis but more likely that a selection process operates to segregate those membrane components required to sustain membrane function from those that are not. A selection mechanism based on function may provide a plausible explanation from the standpoint of cell survival, but it is difficult to account for the apparent selection of other components whose function is more obscure. Polymorphonuclear leucocytes, for instance, possess at least three types of lectin binding sites, one which binds concanavalin A, another binding a different lectin, *Ricinus communis* agglutinin, and a third site which binds both lectins in a linked manner. Oliver et al.[539] found that during phagocytosis there is a loss of total specific lectin binding to the residual plasma membrane which closely parallels the amount of membrane incorporated into the cell. However, only the dual lectin binding site appears to be removed from the cell surface.

There is some indication that microtubular structures in the cytoplasm are involved

in some way in the selection of plasma membrane components internalized during phagocytosis. Ukena and Berlin[540] have shown that pretreatment of phagocytes with drugs like colchicine and vinblastine which disrupt microtubular structures prevents discrimination of various transport sites internalized during phagocytosis. Likewise, treatment of phagocytes with microtubular active drugs leads to an indiscriminant uptake of various lectin binding sites during phagocytosis; however, since the amount of membrane internalized was unaffected, it would seem that intact microtubules are not required for phagocytosis itself. The available evidence strongly suggests that the directional movement of selected components of the plasma membrane is influenced by an interaction with microtubular and microfilamentous structures located in the cytoplasm close to the plasma membrane.[541-546]

*4. Cross-Linking Studies*

Many multienzyme systems, and particularly those located in the inner mitochondrial membrane, are oligomers of two or more different peptide subunits, and the interaction between subunits is presumably required for their functional activity. Not much is known, however, about interactions between other membrane proteins such as whether they exist as free and independent polypeptides or interact with each other randomly, or are associated in a more specific manner. The distribution of certain antigenic and lectin binding sites on the cell surface and the pattern of membrane-associated particles in freeze-cleaved preparations indicate that there are no strong cohesive forces between different membrane proteins. Nevertheless, interactions between different proteins in the plane of the membrane as well as between proteins oriented on either side of the membrane may play some role in the lateral migration of membrane proteins.

One method that has been used to investigate protein-protein interactions in membranes is to cross-link the proteins covalently *in situ* with bifunctional reagents and then examine the type and extent of cross-linking between specific membrane proteins. The procedure can be greatly simplified when cross-linking reactions are reversible because the coupled peptides can be subsequently separated and positively identified. Most of the cross-linking reagents react primarily with amino groups so that proteins can cross-link with amino phospholipids, and this may affect the mobility of the peptides when resolved by electrophoresis. It should also be pointed out that each cross-linking reagent requires a specific alignment between reactive groups on different peptides, and it is conceivable that even oligomeric subunits may fail to interact if no suitable alignments exist even though the peptides may be in close proximity. Such risks can usually be minimized by employing a number of different bifunctional reagents in parallel experiments. Another difficulty is encountered when identifying cross-linked proteins on the basis of enzyme activity or other functional parameter since fixation frequently alters the conformation of these proteins although some correction is possible by comparing the effects of bifunctional reagents with the corresponding monofunctional analogue.

Steck[547] was among the first to investigate the association between different membrane proteins from the controlled use of cross-linking reagents. He treated human erythrocyte membrane preparations with formaldehyde, glutardialdehyde, and several oxidizing agents including o-phenanthroline-$CuSO_4$, a reagent that forms disulfide bridges between peptides, and then extracted the proteins for an examination of the products formed. Under mild conditions, in which excessive reaction was prevented, it was found that certain bands in gel electrophoretograms diminished or disappeared altogether and were replaced by new high molecular weight bands consisting of cross-linked peptides. His general conclusion was that some major proteins of the erythrocyte membrane interact specifically with identical or closely related proteins, while

other proteins did not appear to interact at all. In similar studies of ox erythrocyte membranes, Capaldi[548] found that more extensive cross-linking with glutardialdehyde coupled almost all membrane proteins. Moreover, these highly cross-linked membranes were resistant to disruption by sonication or detergents. By resorting to more drastic procedures such as boiling the membranes in sodium dodecyl sulphate, about 10% of the total membrane protein could be removed, and virtually all of this was glycoprotein. This confirmed the previous observation of Steck[547] that sialoglycoprotein of the erythrocyte membrane is not cross-linked with glutardialdehyde or, for that matter, by a number of other bifunctional reagents, suggesting that electrostatic repulsion between the highly charged polar residues prevents cross-linking.

A refinement of the cross-linking reaction has since been reported by Ji,[549] who used bifunctional reagents with varying distances separating the functional groups from which an estimate of the proximity of proteins in the membrane could be obtained. In human erythrocyte membranes, for example, glycoproteins were cross-linked with dimethyl adipimidate in which the functional groups are separated by a distance of 860 pm but not with dimethyl malonimidate under the same conditions, where the distance is only 490 pm. Examination of the complex, cross-linked with the adipimidate reagent, showed that glycoproteins reacted with each other as well as other membrane proteins. These results indicate that glycoproteins of the erythrocyte membrane do not normally approach within 500 pm of other membrane proteins. Biimidates have been used to investigate protein-protein interactions in a variety of membranes[550-552] as well as the quaternary structure of oligomeric proteins.[553,554] Cross-linking studies have been performed on some functional proteins including $Ca^{2+}$-activated ATPase of rabbit muscle sarcoplasmic reticulum[555] where it was found that the protein associates preferentially into a tetramer; no cross-linked dimers, trimers, or pentamers, etc. were observed. The association of four protein units is consistent with other evidence obtained by freeze fracture of deeply etched membranes[556] and by ultracentrifugation studies.[557] Cross-linking of mitochondrial membranes has been found to inhibit electron transport[558] and reduce mitochondrial ATPase activity.[559] Protein rotation,[466] aggregation,[485] and mobility of lectin receptors[560] is also prevented by bifunctional reagents.

Bifunctional reagents have also been used to explore the interaction between certain amino phospholipids and proteins of the human erythrocyte membrane. Marinetti et al.[561] prepared lipid soluble extracts of membranes cross-linked with 1,5-dinitrofluoro-2,4-dinitrobenzene or 4,4′-difluoro-3,3′-dinitrodiphenyl-sulphone. On hydrolysis these extracts yielded water-soluble derivatives of amino phospholipids. Since about 20% of the total phosphatidylserine and phosphatidylethanolamine of the membrane were cross-linked to membrane proteins whereas only 5% were cross-linked to each other, it was concluded that most of these phospholipids are associated in close proximity to membrane proteins. The development of azido-phospholipid analogues capable of photolytic activation by suitable irradiation should prove useful probes of nearest neighbor associations in membranes.[562,563]

### E. Summary and Critique

The arrangement of phospholipids or galactolipids in a bilayer configuration is a prominent feature of animal and plant membranes. In most biological membranes investigated so far, the lipids appear to be distributed asymmetrically in the bilayer, but the functional significance, if any, of this feature is not known. In reconstitution studies of instrinsic membrane-bound enzymes, for example, almost complete recovery of activity can be achieved with pure phospholipid preparations, suggesting that the lipid requirements for expression of enzyme activity of the protein exposed to either leaflet of the bilayer are the same. Another aspect of lipid asymmetry is that it could

reflect the way membranes are differentiated and repaired by the exchange of lipid molecules from one membrane to another.

Carbohydrates are also assymmetrically arranged, and in the plasma membrane the glycoproteins and glycolipids collectively represent the cell coat. Some of these carbohydrate residues constitute important cell-surface antigens including the ABO blood group substances. The function of most other carbohydrate groups, however, is still obscure.

Membrane proteins are not required for directing or maintaining the structure of membranes, but many are known to possess catalytic activity; more than 75 different enzymes, for example, have been shown to be expressed on endoplasmic reticulum. The identity of many proteins components, nevertheless, is not clear — nor is the way in which they associate with the lipid. Some proteins appear to penetrate completely through the membrane while others do not. The reason why some proteins are capable of rotation about an axis perpendicular to the plane of the membrane while others are comparatively fixed in position is also not known. The significance of lipid fluidity in protein function is also open to conjecture. Changes in the energy of activation of many membrane-bound enzyme processes, for example, have been related to lipid phase transitions either in the bulk lipid bilayer of the membrane of the lipids in the immediate vacinity of the protein. The effect of phase separations and protein aggregation on enzyme function, however, could explain the characteristic discontinuities frequently observed in Arrhenius plots of membrane-bound enzyme processes. The role of lipid fluidity in patching and capping phenomena is still uncertain, especially the role of cytoplasmic structures in these processes. Finally, the existence of multienzyme reactions such as electron transport in mitochondria, endoplasmic reticulum, and chloroplasts have been reasonably well characterized, and the disposition of the individual components describing the relationship between other membrane proteins and the existence of specific interactions between lipids and proteins is the subject more of speculation than of experimental evidence.

## VI. DYNAMICS OF MEMBRANE STRUCTURE AND REGULATION OF FUNCTION

During growth and development, cell membranes are synthesized and differentiated to perform their various functions. Many studies, particularly those using radioisotopic methods, have shown that a large number of membrane components are metabolically labile and turn over at a rate considerably faster than the generation time of the cell. It has been noted that, generally, membrane lipids are more metabolically fluid and turn over at a faster rate than the protein components. The rapid synthesis and degradation of membrane constituents may serve to introduce structural modifications into membranes consistent with changes in their location within the cell and the particular functions they perform. A noteworthy feature in this respect is the apparent need to maintain the appropriate fluidity characteristics within closely defined limits. As we have already noted, the length and presence of *cis* double bonds in the fatty acyl residues associated with the membrane lipids is the primary factor determining membrane fluidity, and the fluidity can be modified by the presence and distribution of cholesterol and intrinsic membrane proteins.

The fluidity of cell membranes has been correlated with numerous biochemical and biological processes including growth, membrane transport, and enzyme activities. Many cells, for example, do not proliferate at temperatures below the bulk phase transition temperature of membrane lipids.[564-567] Thus the optimal growth temperature of *Acholeplasma laidlawaii* is about 36°C provided the membrane achieves a certain degree of fluidity, related to the fatty acid composition of the lipids, above or below

which the rate of growth decreases.[568] The morphology of certain cells including *A. laidlawii* is also affected by membrane fluidity. Cells with highly fluid membranes assume a filamentous form and grow rapidly whereas cells with more rigid membranes tend to round up, swell, and eventually lyse, effects which appear to involve permeability changes in the membrane.

Apart from effects on growth and morphology, changes in membrane fluidity are known to alter the energy of activation of many membrane-associated processes. Abrupt changes in activation energy, observed in Arrhenius plots, over a relatively narrow range of temperature frequently correlated with changes in the physical properties of the membrane. Examples of this behavior include the activity of membrane-bound enzymes,[569-575] active transport of solutes across membranes,[576-578] and mitochondrial respiration.[579] In a study of the transport of galactose into *Escherichia coli*, it was found that the temperature at which the process exhibits a discontinuity in the activation energy coincides with a change in state of monomolecular films of lipids extracted from the membranes.[580] The effect of temperature and lipid phase transitions on a variety of membrane transport processes has been reviewed recently by Cronan and Gelmann,[581] and a discussion of the alternative explanations of the origins of these effects can be found in Reference 582. The relationship between membrane fluidity and other membrane processes such as membrane adhesion, membrane fusion, protein rotation and diffusion, and patch and cap formation has also been reviewed.[461]

## A. Synthesis and Turnover of Membrane Proteins

Membrane biosynthesis and differentiation has been studied largely from the point of view of the mechanism and site of synthesis of the various components. Nearly all cells possess the machinery required for the synthesis of the various protein and lipid components that are found in the constituent membranes. A noteworthy exception is the mammalian erythrocyte, which is incapable of renewing its membrane protein complement and relies upon exchange with plasma lipid fractions for lipid replenishment.

### *1. Site of Synthesis of Membrane Proteins*

Membrane proteins have been classified into two types by Rothman and Lenard[583] on the basis of their disposition within the membrane structure. The location of each particular protein within the membrane is explained in terms of the biosynthetic mechanism. Ectoproteins are defined as those membrane proteins which have a domain residing in the aqueous region of the membrane opposite to that of the cytoplasm from which they were assembled on the ribosome. Typical examples of such proteins include the transmembrane glycoproteins of the plasma membranes of many cells and viral envelopes. Endoproteins, on the other hand, can be adsorbed to the cytoplasmic surface of the membrane, e.g., spectrin and cytochrome c, or can be interpolated into the hydrocarbon region but not completely span the membrane. The later includes many of the electron transport components of the endoplasmic reticulum such as NADPH-cytochrome $b_5$ reductase and cytochrome $b_5$.

The initiation of ectoprotein synthesis is thought to occur on free cytoplasmic ribosomes which generate a signal directing the ribosome, together with its nascent chain, to a receptor site on the endoplasmic reticulum.[584,585] The signal directing the ribosome complex synthesizing the envelope glycoprotein G, of vesicular stomatitis viral envelope to endoplasmic reticulum in a cell-free system appears to be the first 15 to 30 amino acids of the N terminus.[586] Similar N-terminal extensions have been observed on other membrane proteins[587] and a variety of secretory proteins synthesized on membrane-associated ribosomes.[588-590] It is suggested that all membrane proteins that possess these N-terminal sequences are ectoproteins. The nascent chain is extruded

through the membrane of the endoplasmic reticulum, possibly via a specialized ribosome-membrane junction, until the extracytoplasmic sequence of the hydrophilic domain of the protein is completed. At this stage it is thought that the ribosome-membrane junction dissociates allowing elaboration of the C-terminal region to proceed on the cytoplasmic side of the membrane. The attachment of some of the carbohydrate residues to ectoproteins takes place during synthesis of the protein on the rough-surface endoplasmic reticulum[586] while the remainder is glycosylated immediately prior to its appearance in the plasma membrane,[591] and little in the way of a reserve of the complete glycoprotein appears to exist within cells.[592]

In contrast to ectoproteins the endoproteins do not possess the "so-called" signal N-termini, and the nascent polypeptide chain is not extruded through the membrane; the membrane affinity of the molecule is thought to reside in the C-terminal sequences or possibly regions of secondary or tertiary structure that develop during synthesis. Whether or not the ribosomes themselves are associated with the endoplasmic reticulum is problematic. Low and Hallinan,[593] for example, have investigated the site of synthesis of the endoprotein, NADPH-cytochrome c reductase, by comparing the incorporation of labeled amino acid into the enzyme by preparations of rough-surface endoplasmic reticulum and free polysomes. Labeled enzyme was synthesized in both preparations but, on average, a fourfold higher rate was observed on unbound polysomes. The rate of enzyme synthesis in some preparations of rough-surface membrane was appreciable, and they could not exclude the possible contamination with free polysomes or the attachment of these to the membrane by amino acid sequences nearing completion.

Certain proteins of mitochondrial and chloroplast membranes cannot be classified on the basis of the scheme described by Rothman and Lenard[583] because some polypeptide components are coded by mitochondrial or chloroplast genes and synthesized on the ribosomes present in these organelles whereas the remaining polypeptides that are required for the oligomer are coded by nuclear genes and synthesized on cytoplasmic ribosomes. The different mechanisms of synthesis at organelle and cytoplasmic sites have been exploited to identify the origin of particular peptides in oligomeric proteins. The synthesis of mitochondrial ATPase and cytochrome oxidase in yeast has been examined by the use of selective inhibitors of protein synthesis.[594] Mitochondrial ATPase is known to consist of ten non-identical subunits six of which are coded by nuclear genes and synthesized on cytoplasmic ribosomes, and four are synthesized on mitochondrial ribosomes and presumably coded by mitochondrial DNA. Similarly, cytochrome oxidase consists of seven subunits four of which are of cytoplasmic origin and three of which are mitochondrial. Other studies of the incorporation of radioactive formate (the initiator for mitochondrial protein synthesis is formylmethionyl (f-Met)-tRNA) have shown that peptide synthesis at the two sites is closely coordinated and geared to the prevailing requirements of the cell.[595]

### *2. Rate of Synthesis and Degradation of Membrane Proteins*

Radioisotope studies with labeled amino acids in pulse-chase type experiments usually show a considerable delay between synthesis of plasma-membrane proteins and their ultimate appearance on the cell surface. The specific activity of total labeled rat liver proteins in a pulse-labeled animal, for example, remains relatively constant over a period of about 4 to 5 hr, but the distribution of radioactive proteins between different subcellular membranes changes with time.[596] Thus the specific activity of proteins of the smooth endoplasmic reticulum decrease exponentially after the initial pulse, and there is almost a corresponding increase in proteins of the plasma membrane. More detailed studies of the specific radioactivity of individual membrane peptides have been

reported which show an increase in all peptides during the first 2 hr following an amino acid pulse, but peptides of molecular weight less than 50,000 had a higher specific radioactivity than heavier fractions.[597] It was suggested that individual protein components could be incorporated into the plasma membrane at different rates and possibly by different mechanisms. It should be emphasized, however, that only the appearance of radioactive peptides were measured, and loss of radioactivity due to membrane protein turnover are assumed to be negligible during the course of such measurements.

Turnover studies of protein and lipid of endoplasmic reticulum of rat liver have been compared following a single intraperitoneal injection of radioactive precursors.[598] The rate of a decrease of radioactivity in the total protein and lipid fractions suggested that the lipids turn over at a faster rate than the proteins; no differences in turnover of either lipids or proteins were observed between rough and smooth surface endoplasmic reticulum. The observations were extended to include the activity of two membrane-bound enzymes, reduced NADP:ferricytochrome c oxidoreductase and cytochrome $b_5$. Specific enzyme and radioactivities were determined on each protein after extraction and purification from the membrane, and it was found that the turnover rate of the reductase was considerably faster than cytochrome $b_5$, which had a turnover rate of approximately the same as the average half-life of proteins of the membrane.

A number of studies suggest that the rate at which new proteins appear in the plasma membrane of various cells does not vary greatly with the rate of growth or functional activity of the cell. The degradation or removal of components from the surface of mouse fibroblasts, for example, appears to be relatively faster in quiescent cells compared with rapidly growing cells, indicating that membrane components are conserved in the differentiation of new membranes during periods of increased activity.[599] Studies of cells undergoing phagocytosis also indicate no significant increase in the rate of synthesis of plasma-membrane components compared with other subcellular membranes or even when compared with resting cells. Components of the plasma membrane of phagocytosing cells are replaced even in the presence of inhibitors of protein synthesis,[600] suggesting that there is an intracellular reserve of at least some plasma-membrane proteins or possibly reflecting a more efficient recycling of internalized membrane components. The rate of appearance of plasma-membrane components throughout the cell growth of mouse and hamster tissue culture cells has been reported.[601] In general, protein and lipid components appear to be inserted continuously during interphase although the incorporation of carbohydrate is more variable. Certain plasma-membrane-specific components such as $(Mg^{2+})$-ATPase, 5′-nucleotidase, amino acid transport proteins, and histocompatibility antigens all inserted during early interphase whereas the activity of other membrane-bound enzymes varies through the cell cycle. It should be noted, however, that enzyme inactivation or rapid degradation may have obscured the synthetic rate of these components.

## B. Origin and Turnover of Membrane Lipids

The predominant site of *de novo* synthesis of the major phospholipid classes is the endoplasmic reticulum. Enzymes involved in phosphatidylcholine synthesis, shown by the ability to incorporate CDP-choline into phospholipid, are located in the endoplasmic reticulum. In liver, both rough and smooth membrane fractions appear to incorporate the precursor at about the same rate.[602] Mitochondria are also capable of synthesizing certain phospholipids such as cardiolipin, phosphatidic acid, and phosphatidylglycerol but not other phospholipids.[603] Synaptosomal membranes from guinea pig brain have also been shown to synthesize phospholipid.[604]

### *1. Pathways of Phospholipid Metabolism*

Isotopic experiments have provided convicing evidence that phospholipids of all

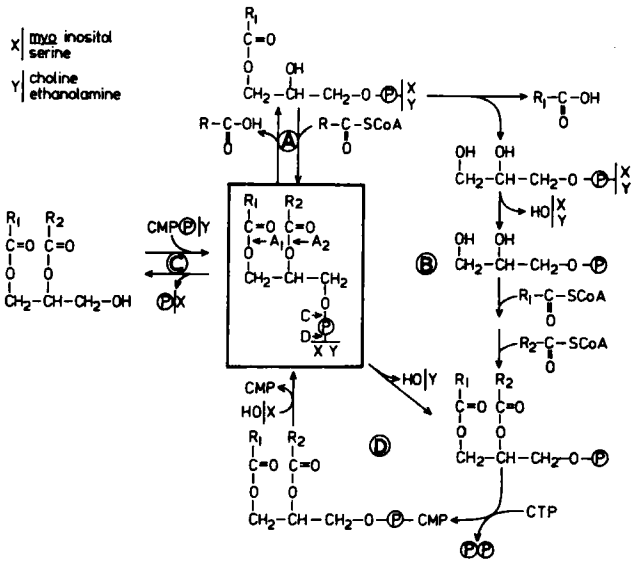



FIGURE 17. Major metabolic routes of phospholipid turnover. The bonds of the phospholipid molecule susceptible to attack by phospholipase enzymes $A_1$, $A_2$, C, and D are shown. The combined action of $A_1$ and $A_2$ (deacylation of the phospholipid) leads to pathway B in which the base or myoinositol, X, or Y is subsequently removed to form *sn*-glycerol-l-phosphate. Subsequent reacylation in two stages yields phosphatidic acid. Phosphatidic acid may be derived directly from phospholipids by the action of phospholipase D. Phospholipases similar to the D type are responsible for base exchange reactions as distinct from pathway D, which is not generally associated with animal cells. Hydrolysis of phospholipid by phospholipase C produces a diglyceride which represents the route of synthesis (pathway C) of choline and ethanolamine phosphatides.

membranes turn over at a rapid rate, and in most cases this turnover is substantially greater than that of the proteins. Many, but not all of the chemically distinct groups of phospholipid molecules — that is, the fatty acids, glycerol, phosphate, and amine-containing alcohol or *myo*inositol — are linked by ester bonds. Synthesis and degradation of these phospholipids involve condensation and hydrolytic reactions, respectively. Some of the more important of these reactions are shown in Figure 17. More detailed reviews of these pathways can be found elsewhere,[605-608] including the synthesis of long-chain fatty acids.[609-611] We have divided phospholipid synthesis and degradation into three categories: in A (Figure 17), the essential amphipathic character of the molecule is retained since the pathway involves merely hydrolysis of one hydrocarbon chain followed by recondensation with another — this is referred to as acyl exchange and represents a partial turnover of the lipid; in pathways B and C, degradation to water soluble compounds such as glyceryl and phosphoryl esters is involved. The difference between these two routes lies in which bond of the phospholipid molecule is first hydrolyzed. Pathway B begins with the removal of an acyl chain (as in pathway A) followed by removal of the second acyl chain followed by degradation of the resultant water-soluble products. Degradation by pathway C begins with removal of a phosphoryl base or (cyclic) phosphoryl inositol. In no case does synthesis proceed by a reversal of degradation; rather, it is a separate process, involving a condensation of an unsubstituted diacylglyceride with a cytidine diphosphate derivative (CDP-y) or,

vice versa, condensation of cytidine diphosphodiglyceride with unsubstituted X (Figure 17). In general, it has been assumed that degradation in animal cells involves pathways A and B[612] rather than C, and this has recently been confirmed in a comparative study of several cell types.[613] Pathway C operates significantly only with phosphatidylinositol; the mechanism is one of group transfer rather than of hydrolysis in the sense that cyclic phosphoryl inositol is formed.[614] In the case of sphingolipids, rather little is known of the synthesis and degradation of the acyl side chain. In principle, a pathway such as that of A (Figure 17) might operate, but the fact that an amide, not an ester, bond is involved is likely to introduce a different degree of specificity. Metabolism of the polar group, on the other hand, has received considerable attention. A C-type enzyme specific for sphingomyelin has been reported.[615] and in the case of glycolipids, both addition and removal of successive glycosyl residues is catalyzed by specific enzymes.[616] As with phospholipids, turnover is extensive, since absence of a particular degradative enzyme[617,618] leads to an accumulation of precursor glycolipids, to the point of severe clinical manifestations.[619-621] In one instance at least, absence of a synthetic enzyme leads to the same result.[622]

The turnover rate of individual lipid classes varies greatly; sphingomyelin, for example, is more stable than phosphatidylcholine, and the turnover rate of sphingomyelin in membranes tends to be much slower. Of all the major phospholipid classes, phosphatidic acid and phosphatidylinositol turn over in cell membranes at an appreciably faster rate than any of the other phospholipid classes. The metabolic lability of phosphatidic acid may be ascribed to the fact that it occupies a key position in the synthesis of most other phospholipids, but phosphatidylinositol is exceptional. Not only is the pathway of synthesis unique in the sense that *myo*inositol is attached to cytidinediphosphodiglyceride, but phospholipase c-type enzymes are present in most tissues that specifically degrade the lipid[623] (see Section VI.B.3). The nature of the acyl chain also appears to be important in turnover rates; diacylglycerophosphatides with short unsaturated hydrocarbon chains are more susceptible to phospholipases A and C and turn over faster than molecules containing long saturated fatty acyl residues. This difference may be due to the decreased hydrophobicity of phospholipids with short unsaturated chains, which allows them to take up a more exposed position on the membrane surface. The proximity of phospholipids to intrinsic membrane proteins may also be a factor affecting susceptibility to phospholipase attack.

Since phospholipids are synthesized mainly in the endoplasmic reticulum,[624] it is clear that intact phospholipid molecules must be transported from this site and inserted into other membranes located elsewhere in the cell. The movement of subcellular membranes and the associated chemical modifications connected with the transport and processing of proteins destined for secretion from many cells has been well documented.[625,626] As a consequence of these membrane movements, phospholipids (as well as membrane proteins) may be relocated from their site of synthesis. During this translocation process or upon reaching their destination, phospholipids may be subject to partial turnover as described above. Thus, exchange of fatty acyl residues or polar groups between molecules modifies the character of the lipids. (We refer to these reactions as partial turnover because the intermediate of the reactions, e.g., lysophosphatide or phosphatidic acid, do not lose their character as phospholipids). Membrane-bound enzymes such as phospholipase A have been identified in a number of subcellular structures including lysosomes,[627] chromaffin granules,[628] mitochondria, and microsomes[629,630] and they are likely to be involved in these exchange-type reactions. For a review of the dynamics of phospholipids in the plasma membranes of cells, see Reference 631.

*2. Exchange of Phospholipids Between Membranes*

Unlike intrinsic membrane proteins, the phospholipids of subcellular membranes appear to be readily exchangeable from one membrane to another. The mediation of these exchange reactions by soluble proteins was first observed in studies of a system consisting of rat liver microsomes containing radio-isotopically labeled phospholipids mixed with an unlabeled mitochondrial suspension.[632-634] The transfer of labeled phospholipids to the mitochondria was considerably enhanced by addition of a supernatant fraction from the tissue homogenate. Subsequently it was shown that transfer of phospholipids from mitochondria to microsomes could be accomplished, suggesting that the transfer process consisted of an exchange-type reaction rather than a net movement of molecules from one membrane to another.

Specific phospholipid-exchange proteins appear to be required for each of the major phospholipid classes, and the protein responsible for phosphatidylcholine exchange has been identified in a variety of mammalian tissues. The protein has been purified from beef liver, and it was shown to catalyze a rapid exchange of phosphatidylcholine between endoplasmic reticulum and mitochondria; a high degree of specificity was inferred because other phospholipids such as phosphatidylethanolamine and phosphatidylinositol were not transferred.[635] Monomolecular film experiments with phosphatidylcholine have shown that the exchange protein is capable of transferring phospholipid between lipid interfaces with one molecule of phosphotidylcholine always associated with the protein.[636]

*3. Trigger Mechanisms for Lipid Degradation*

The enhanced turnover of certain membrane lipids has been shown in some circumstances to be coupled to a physiological stimulus. Such effects are superimposed on the normal differences that are observed in metabolic turnover rates between the various lipids present in membranes. Thus, sphingomyelin is generally more stable than phosphatidylcholine,[637,638] and this is true for turnover in vivo.[639-641] We have already noted the importance of the type of fatty acid associated with the membrane lipid on turnover rates, and these effects appear to be due to differing susceptibility to phospholipases A and C.[642] The proximity of membrane proteins has also been used to explain why some 25 to 40% of phospholipids are resistant to bacterial phospholipase C.[643-646] The proteins are believed to offer some kind of protection to the substrate molecules,[647] but pretreatment of membranes with trypsin does not always increase susceptibility of phospholipids to enzyme digestion.[648]

The uptake of inorganic phosphate and other precursors into phosphatidylinositol and phosphatidic acid has been known for some time to be associated with specific stimulation of a variety of different tissues. Thus phosphatidylinositol synthesis is reported to increase in polymorphonuclear leucocytes phagocytosing polystyrene granules,[649,650] in lymphocytes treated with phytohaemagglutinin[651-653] and in serum-mediated relief of density-dependent inhibition of growth.[654] Hormonal stimulation of target tissues elevates $^{32}Pi$ incorporation into phosphatidylinositol of adipose tissue stimulated by insulin,[655] thyroid gland treated with pituitary thyrotropin,[656] pineal gland stimulated with a variety of phenethylamines and local anesthetics,[657] and rat heart following adrenaline administration.[658] Similar effects have been reported in nervous tissue treated with acetylcholine,[959] low concentrations of chlorpromazine and other tranquilizing drugs,[660,661] local anesthetics,[662] noradrenaline,[663] or following electrical stimulation.[664,665] There is generally no marked increase in the proportion of phosphatidylinositol in stimulated tissues, suggesting that incorporation of precursors reflects enhanced turnover of the phospholipids. Furthermore, phosphatidylinositol turnover is elevated generally within a few minutes of application of the primary stimulus tending to link degradation and synthesis of the phospholipid with subsequent

cellular responses. The connection between the primary stimulus and a phospholipase C-type enzyme responsible for phosphatidylinositol degradation in most tissues has been the subject of a recent review.[666]

The precise function of increased phosphatidylinositol turnover in stimulated tissues has not yet been defined. The possible general effects on the structure and function of cell membranes has been considered, especially in regard to regulating the influx or efflux of metabolites and ions[667] or macromolecules incorporated by phagocytosis or pinocytosis.[668] An alternative mechanism whereby one of the products of phosphatidylinositol degradation, *myo*inositol 1:2 cyclic phosphate, functions in some "second messenger" capacity has been suggested,[669,670] but no direct evidence for such an effect has been identified.[671]

Another situation where phospholipid turnover is stimulated concerns the synthesis of prostaglandins. Membrane phospholipids represent the major reserve of arachidonic acid which serves as the substrate precursor for prostaglandin synthesis in most tissues. It was proposed some time ago that phospholipase A was likely to be the triggering enzyme for regulating the production of prostaglandins by its action in releasing the fatty acid from membrane phospholipids.[672] A number of studies have been reported on the relationships involved between arachidonic acid metabolism and prostaglandin synthesis, especially in blood platelets. It has been shown, for example, that incubation of human platelets with arachidonic acid results in an appreciable incorporation of the fatty acid into various membrane phospholipids but predominantly into the phosphatidylcholine and phosphatidylinositol fractions. The release of a significant proportion of this fatty acid and its conversion into oxygenated products can be accomplished by incubating platelets briefly with agents such as thrombin which cause platelet aggregation.[673-675] It has been suggested that the same deacylating enzyme-producing lysophosphatides required for the incorporation of arachidonic acid via the Lands pathway[676] is also responsible for the release of the fatty acid upon stimulation by platelet aggregating factors. To account for the selective turnover of the fatty acids of phosphatidylcholine and phosphatidylinositol, it is necessary to postulate that the enzyme shows a high degree of specificity for these phospholipids or that they are more readily available for enzyme attack. Recent studies have shown that the 1-acyl-*sn*-glycero portion of the phospholipid assumes a more critical role in determining the affinity of phospholipase A enzymes for their potential substrates,[677] but acylation of lysoglycerophosphatides is a reaction dependent upon the polar head group of the molecule.[678] In the thyroid gland, the principal phospholipid involved in arachidonic acid turnover appears to be phosphatidylinositol.[679] The specific incorporation of arachidonic acid into phosphatidylinositol was found to be three-fold greater than into other phospholipids such as phosphatidylcholine, and its release appeared to be mediated by a specific phospholipase $A_2$, the activity of which was stimulated by thyrotropin through a calcium-dependent process in which cyclic-AMP was not involved.

## C. Biochemical and Genetic Control of Membrane Fluidity

A number of different methods have been used to alter the fluidity of membranes of living organisms in order to investigate the relationship between fluidity and particular membrane functions. Cell membranes of homeothermic organisms provide only limited scope for such manipulations which are restricted largely to nutritional means, but substantial changes in membrane fluidity of poikilothermic organisms can be achieved by a number of experimental devices.

### *1. Isolation and Studies of Genetic Lipid Auxotrophs*

The isolation of lipid auxotrophs of *Escherichia coli* and *Saccharomyces cerevisiae* which lack particular enzymes concerned with lipid synthesis have been particularly

useful in studies of lipid fluidity.[680,681] Two mutants of *E. coli* deficient in enzymes responsible for the synthesis of unsaturated fatty acids have been described,[682-684] and neither will survive beyond one generation unless provided with a supplement of monoenoic fatty acids or a suitable substitute.[685] Studies of another fatty acid auxotroph unable to synthesize either saturated or unsaturated fatty acids[686] has indicated that saturated fatty acids in addition to unsaturated fatty acids are required to sustain membrane function. Thus, membrane permeability to $K^+$ and o-nitrophenyl-thiogalactoside increases markedly when the proportion of saturated compared to unsaturated fatty acids decreases to less than about 15% of the total membrane fatty acid content.[687]

In yeasts, as in other eukaryotes, long-chain saturated fatty acyl-CoA derivatives are desaturated in the $\omega^3$, $\omega^6$, or $\omega^9$ positions by reactions requiring oxygen. Growth under anaerobic conditions sustained by fermentation reactions can therefore render such organisms deficient in unsaturated fatty acids. The effects on functions depending on membrane fluidity in anaerobically grown cells, however, are restricted to those that do not depend on respiration. Studies of lipid-supplemented desaturase mutant strains of yeast that would otherwise be unable to grow even under aerobic conditions[688] have revealed that mitochondrial functions in fact are particularly sensitive to alterations in membrane fluidity.[689] The particular lesion associated with a decreased membrane fluidity has been identified as an increased permeability of the inner mitochondrial membrane to protons, a situation that effectively uncouples oxidation from phosphorylation of ADP.[690] Mutant strains of yeast with specific defects in enzymes required for sterol[691] and saturated fatty acid synthesis[692] have also been reported.

*2. Alteration of Membrane Fatty Acids by Nutritional Supplementation*

The type of fatty acids incorporated into newly synthesized membrane lipids depends to a certain extent on the fatty acids supplied from the diet and their subsequent biochemical modifications as well as endogenous synthesis of long-chain fatty acids. Apart from those dietary fatty acids incorporated into membrane lipids in an unmodified form, the nature of the remaining fatty acid pool is reflected by the relative rates of fatty acid synthesis and the rate at which fatty acids are desaturated. It has been shown that fatty acid desaturase reactions can be markedly influenced by diet. When fasted rats are refed on a diet rich in carbohydrates and low in fat, for example, the activity of stearyl CoA desaturase of the liver can be increased to levels 10- to 20-fold greater than normally fed individuals.[693]

The fatty acid composition of rat tissues have been altered by nutritional methods, and this has led to changes in the activity of enzymes of sarcoplasmic reticulum[694] and the erythrocyte membrane[659-697] as well as other membrane-bound enzymes.[698] Supplementation by either fatty acids or cholesterol have been equally effective in producing these changes, suggesting that membrane fluidity rather than specific interactions between membrane components is of paramount importance. Supplementation of cells in tissue culture, particularly in the presence of drugs that interfere with lipid synthesis, provides a considerably wider scope for manipulating membrane fluidity.This has been achieved in two mouse cell lives, L. M. and $BHK_{21}$ supplemented with either Tween fatty acid esters[699] or fatty acids complexed to bovine serum albumin[700] in the presence of desthiobiotin, a drug that inhibits endogenous fatty acid synthesis.

*3. Drugs Affecting Lipid Biosynthesis*

A number of drugs which inhibit lipid synthesis in bacterial systems have been described and their effects on membrane lipid composition and fluidity examined. The antibiotic cerulenin, for example, inhibits synthesis of all fatty acids in *E. coli* and supplementation with both saturated and unsaturated fatty acids are required for growth.[701] Inhibition of unsaturated fatty acid synthesis in bacteria by 3-decyonyl-*N*-

acetylcysteamine has been shown to inhibit growth, which can be restored by supplementing oleic but not palmitic acid to the growth medium.[702] The drug produces an overall decrease in fatty acid synthesis, but the proportion of saturated fatty acids increases markedly. The synthesis of various phospholipid classes by *E. coli* is inhibited to differing degrees by penethyl alcohol.[703] In the presence of certain concentrations of the drug phosphatidylethanolamine, synthesis is inhibited to a greater extent than phosphatidylglycerol, but disphosphatidylglycerol synthesis is unaffected, leading to marked changes in the proportion of phospholipid classes represented in the membranes. Examination of the fatty acids associated with these phospholipids has shown that the proportion of unsaturated fatty acids (predominantly *cis* vaccenic and palmitoleic acids) compared to the saturated fatty acids increases, suggesting that membrane fluidity is altered by exposure to the drug.

*4. Temperature Acclimatization*

Living organisms are able to adapt themselves to a greater or lesser extent to changes in environmental temperature. The underlying biochemical changes associated with temperature acclimatization may be facilitated by the existence of isoenzymes with characteristics varying with temperature or enzymes displaying different affinity for their substrates. The participation of certain regulator proteins responsible for altering the synthesis or degradation of cellular components including membrane lipids have also been considered as possible agents mediating adaption at the biochemical level.The scope for acclimatization of any particular organism to different environmental temperatures is usually well defined beyond which viability is lost. It should be noted, nevertheless, that specialized structures representing quiescent stages in the growth cycle such as spores may survive temperatures that would otherwise destroy the vegetative organism. This implies that changes in temperature must be gradual in order to permit differentiation of these structures. Indeed there is abundant evidence that rapid chilling from temperatures greater than about 25°C to 0-2°C, in contrast to slow cooling, leads to cold shock of protozoa,[704] algae,[705] and bacteria.[706-708] Spermatozoa of a number of animals are also susceptible to cold shock, and this has been related to the lipid content of the constituent membranes.[709] Lovelock[710,711] has suggested that a decrease in the ratio of phospholipid to cholesterol of the human erythrocyte membrane results in instability and permeability changes associated with cold shock.

In general poikilothermic and heterothermic organisms are better able to acclimatize on a cellular level to extremes of temperatures than are homeothermic animals. Observations of cold-adapted microorganisms,[712-714] plants,[715-718] and animals[719-721] suggest that the proportion of unsaturated fatty acyl residues of membrane lipids increases to compensate for low-growth temperatures thereby preserving the membrane in a fluid condition[580,722-723] necessary to sustain the activity of a variety of membrane-bound enzymes.[724,725] Recent studies of a thermotoleant strain of the protozoan *Tetrahymena pyriformis,* which responds to varying temperatures by altering both the degree of unsaturation of membrane phospholipids and the relative proportions of the differnet phospholipid classes, have been particularly useful in identifying the mechanisms responsible for controlling membrane fluidity.[726] The suggestion that membrane fluidity is preserved within narrow limits by a metabolic feedback mechanism[727] has been supported by the discovery that the activity of membrane-bound fatty acid desaturases of the organism are controlled by the physical properties of endoplasmic reticulum. Thus, cells supplemented with linoleic acid to increase membrane fluidity showed reduced fatty acid desaturase activity,[728] whereas a decrease in membrane fluidity brought about by a reduction in temperature from 39.5°C to 15°C resulted in a relative increase in desaturase activity.[729] It is of interest that reconstitution experiments in which com-

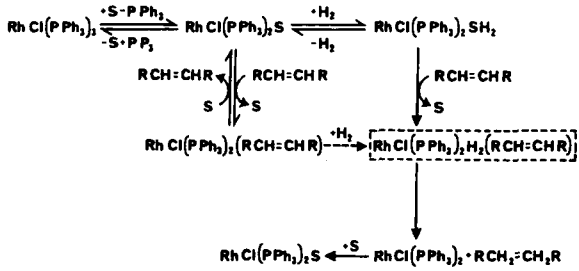


FIGURE 18. Reaction sequences involved in the hydrogenation of olefinic groups of membrane lipids catalyzed by triphenylphosphine chlororhodium (I). Solvent, S, displaces one of the triphenylphosphine ligands of the catalyst complex. This, in turn, may be displaced by an olefin to form a complex, but subsequent hydride formation leading to hydrogenation is uncertain. The formation of the hydride complex appears to be the preferred route, which then interacts with the unsaturated substrate followed by hydrogen transfer. (Adapted from Reference 554).

ponents of the fatty acyl CoA desaturase complex, NADH-cytochrome $b_5$ reductase, and cytochrome $b_5$, have been incorporated into dimyristoyl phosphatidylcholine bilayers, indicate that a fluid bilayer is required to facilitate protein-protein interactions required for electron transfer between the components.[730] These two apparently conflicting observations may be reconciled if the complexity of the hydrocarbon components of the endoplasmic reticulum is considered since only partial crystallization of membrane lipids will result from cooling the membrane, which will result in aggregation of the desaturase components into remaining fluid regions. This process has been described in detail above (see Figure 14).

### *5. Modulation of Membrane Fluidity by Direct Hydrogenation*

Because membrane fluidity is determined to some extent by the unsaturated fatty acyl chains of membrane lipids, the saturation of these olefins should provide a convenient method of manipulating membrane fluidity *in situ*. Conventional methods of hydrogenating natural oils with catalysts such as nickel, copper, platinum, or palladium cannot be applied to membrane systems because contact between substrate molecules of the bilayer and the surface of the metal is unlikely to take place. The use of certain homogeneous catalysts however, appears to be much more promising in this regard. These catalysts consist of complexes formed from the atomic metal. This means that all the metal atoms of the catalysts are putative catalytic centers making them much more efficient in terms of the amount of catalyst required to sustain a given rate of hydrogenation. Furthermore, the ligands associated with the metal can be designed to allow the catalyst to partition from the aqueous medium into the hydrophobic region of the lipid bilayer, where orientation about the unsaturated hydrocarbon residues can lead to selective hydrogenation of the bonds.

A method for introducing a homogeneous catalyst into membrane systems and then hydrogenating the double bonds of the phospholipid has been devised.[731,732] A catalyst developed by Wilkinson and co-workers[733,734] for the hydrogenation of alkenes and active at ambient temperatures and pressures, namely, chlorotris-(triphenylphosphine) rhodium (I) and its sulphonated derivative, were used in these experiments. The chemistry of the hydrogenation process for this catalyst complex is illustrated in Figure 18. Briefly, this consists of an activation of molecular hydrogenation then activation of the substrate followed by the transfer of hydrogen to the olefin. The last step probably

takes place in two stages, first by formation of an alkyl complex between the hydrocarbon and the catalyst, then a second rapid hydrogen transfer step to given the alkane.

The manipulation of membrane fluidity by homogeneous catalytic hydrogenation may have future potential for the study of many cell systems where membrane fluidity has been linked to cell functions, e.g., membrane transport, replication rate, cell movement, membrane fusion, etc. It should also be useful for studying excitable membrane systems which are known to contain significant proportions of highly unsaturated phospholipids, some of which contain five or six double bonds. Thus it should be possible to introduce phase transitions into a variety of biological membranes, and studies of hydrocarbon chain conformation on particular biological functions should be achieved without resorting to extraction and reconstitution techniques.

### D. Summary and Critique

Cell membranes are, in general, dynamic structures in the sense that the constituent molecules are continually undergoing metabolic alterations and degradation and are being replaced so that the overall composition of the membrane at any point in time remains relatively unchanged. The purpose of this flux is not fully understood, but it is likely to be involved in membrane biosynthesis and differentiation. The site of synthesis of individual membrane proteins and lipids has been established as primarily the endoplasmic reticulum, but some components are synthesized elsewhere, e.g., cytoplasmic polysomes, mitochondria, and chloroplasts. The precise way in which the synthesis of the different membrane components are regulated and the methods whereby they are directed and inserted into the membrane at their ultimate location is unknown. The fusion of partially differentiated membrane vesicles is probably concerned in this process, but it is not known what recognition mechanisms are present to direct these events.

Attempts have been made to explain the asymmetry of protein distribution in membranes and the maintenance of this arrangement on this basis of biosynthetic mechanisms. The asymmetry of disposition of lipids, however, cannot be accounted for so easily on the basis of the mechanism of synthesis. Furthermore, the purpose of this arrangement in regard to the function of the membrane is also obscure compared with many membrane proteins whose vectoral functions are now well established.

The metabolic turnover of membrane lipids is usually at a rate somewhat faster than the proteins. Modifications of the lipids *in situ* are known to take place by such processes as acyl and base exchange. The significance of these reactions is not yet fully appreciated although certain membrane functions may possibly be related to these processes. The enhanced turnover of phosphatidylinositol either by phospholipase C-type reactions or deacylating enzymes has been observed in many cells and tissues, but the way turnover is regulated and coupled to trigger mechanisms is unknown. Turnover of the acyl residues of membrane lipids appears to be important in controlling the fluidity characteristics of the membrane. Why fluidity is different in different membranes is possibly related to the particular functional properties of the membrane, but how this is controlled and directed via lipid and fatty acid biosynthesis has not yet been fully explained. The maintenance of membrane fluidity has been related to the genetic and biochemical capacity of cells to synthesize the appropriate lipids or to incorporate specific fatty acids into membrane lipids from dietary sources. Furthermore, there is some adaption to environmental conditions, particularly in poikilothermic individuals, that appears to maintain membrane fluidity within certain limits.

The importance of membrane fluidity on a variety of membrane-associated processes is well established. The manipulation of membrane fluidity, especially by methods that can be adapted for clinical applications, seems to provide a promising means of regulating membrane function and hence controlling the behavior of cells.

## VI. CONCLUSION

Beginning from the complex and distinctive chemical composition of different cell membranes it is hardly surprising that the detailed organization of the molecular components show considerable variation. Nevertheless, the propensity of membrane lipids to form bimolecular structures in aqueous systems and the almost universal existence of this arrangement in membranes constitutes the primary level of organization in membranes. There is now convincing experimental evidence to show that the lipid constituents of the erythrocyte membrane are arranged asymmetrically in the structure, and preliminary studies of other membranes suggests that this may be a general feature of biological membranes. The protein component of membranes shows considerable variation both in type and relative proportion of each protein, particularly in different stages in differentiation of the same membrane. The asymmetric disposition of proteins in membranes has been repeatedly demonstrated, and the principles governing the interaction of proteins with lipid bilayers is now fairly well understood. Thus proteins may be simply adsorbed to the surface of the lipid bilayer while other proteins can be completely interpolated into the lipid hydrocarbon region.

The interaction between different membrane components such as protein-protein and protein-lipid interactions and the effect of these contracts on membrane function is receiving considerable attention. The recognition that lipid mesomorphism can affect the way membrane lipids and intrinsic proteins are arranged in the structure and how this property affects the mobility of individual constituents and, in many cases, their functional activity has provided considerable insight into the structure of membranes and their related functions. It is clear that there will be continued interest in relating the organization of membrane components with particular cellular functions as more detailed information becomes available. Furthermore, the possibility of controlling the behavior of cells by modulating the fluidity of cell membranes, thereby altering the organization and properties of cell membranes, is a promising new development that is likely to have considerable future applications.



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