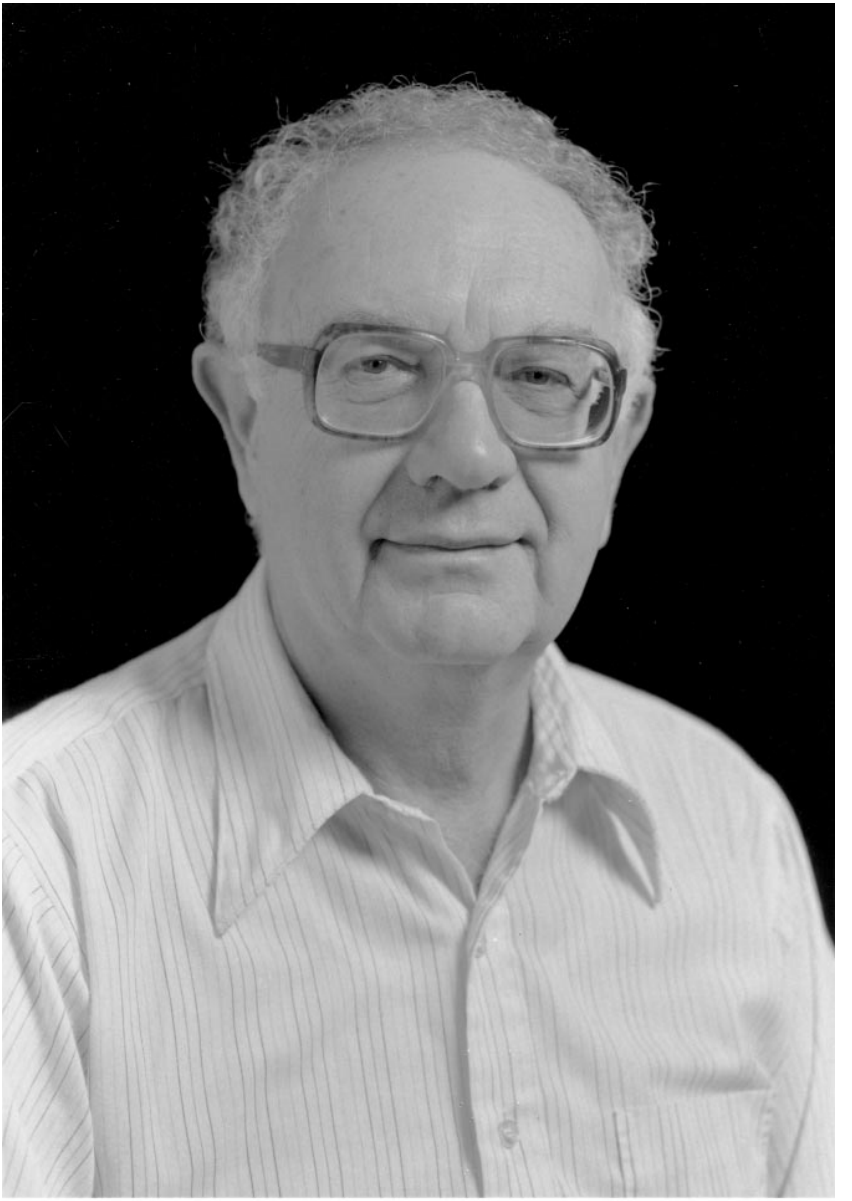

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SOME EARLY HISTORY OF MEMBRANE MOLECULAR BIOLOGY

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■ **Abstract** This article is mostly about the beginnings of the molecular biology of membranes, covering the decade 1964–1974. It is difficult to read (or write) this article because of a sense of déja vu. Most of the material in it is considered commonplace today, having been established experimentally since then. But at the time this work was begun, practically nothing was known about the molecular structure and the mechanisms of the functions of membranes. This situation existed because no membrane proteins of the kind I called integral had as yet been isolated in a pure state, and therefore none had had their amino acid sequence determined. The first integral membrane protein to be so characterized was human erythrocyte glycophorin, in 1978. It was the use of the thermodynamic reasoning that had been developed for the study of water-soluble proteins, together with the information from several key experiments carried out in a number of laboratories during the early decade, that led us to the fluid mosaic model of membrane structure in 1972. Without direct evidence to confirm the model in 1971–1972, my colleagues and I nevertheless had the confidence in it to pursue some of the consequences of the model for a new understanding of many membrane functions, which I present here in some detail. Finally, I discuss two recent high-resolution X-ray crystallographic studies of integral proteins to ask how well the structural and functional proposals that we derived from the fluid mosaic model fit these remarkably detailed X-ray results.

INTRODUCTION

I first became interested in the molecular structure of biological membranes in 1964, coming from a research background involving the physical chemistry of water-soluble proteins. I soon found that my ignorance about membranes was paralleled by the generally rudimentary state of knowledge of the subject, particularly where membrane proteins were concerned. No characteristic membrane protein had yet been isolated and its amino acid sequence determined by 1964; this first occurred in 1978. (This was largely because membrane proteins are not soluble in water). From 1964 to early 1971, when I published a long article on the

lipid-protein mosaic model of membrane structure (1), which was extended to include the fluid character of the mosaic in the *Science* paper of early 1972 (2), my evolving picture of membrane structure and corresponding functions was therefore largely thermodynamic and theoretical. In 2003, all of that work seems remote and old hat, largely confirmed and greatly extended by a tremendous amount of experimental work in the intervening years. It is this now largely forgotten early period, however, that is the main subject of this memoir.

MEMBRANE STRUCTURE IN 1964

Protein-Lipid Ratios

The ratio by weight of membrane-associated proteins to lipids varies from about 1.0 for the red blood cell to 3 for mitochondrial membranes. The only marked exception is myelin, which is an insulating rather than a conducting membrane: This has a ratio of only 0.23. Proteins not only constitute the major component of most membranes but also carry out most of the functions of membranes. Their structures are therefore of primary concern.

Lipid Structure

The experiments of Gorter & Grendel in 1925 (3) were carried out by measuring the surface area on a Langmuir trough of a monolayer of lipid extracted from a known number of red blood cells. They concluded that the amount of lipid was equivalent to twice the surface area of the cells and that the lipids were therefore organized as a continuous bilayer around the cell. This work is referred to today as the source of the lipid bilayer concept and is widely accepted. However, for technical reasons, these experiments turned out to be faulty, and when done correctly (4), the area occupied by the monolayer of lipids in the trough was nearly the same as, rather than double, the cell surface area. The lipid is therefore organized either as a monolayer around the entire cell (which is thermodynamically unsound), or as a bilayer covering only roughly half of the total surface area of the red blood cell. We return to this important fact below.

Electron Microscopy

Conventional transmission electron microscopy of fixed, stained, and plastic-embedded cells revealed that most types of membranes exhibited a similar railroad-track-like appearance (5). The distance between the outer edges of the track was about 90 Å, and if the lipid was regarded as a Gorter-Grendel continuous bilayer of about 70 Å average thickness, this result was interpreted (5, 6) to mean that the membrane protein was largely in the form of two continuous layers of unfolded protein of 10 Å thickness on both sides of the lipid bilayer (Figure 1*A*). This came to be known as the unit-membrane, or the Davson-Danielli-Robertson (DDR) model, and was the generally accepted view of membrane structure in 1964.

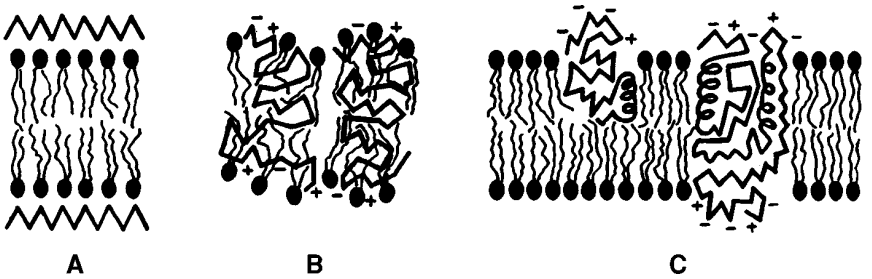


Figure 1 Schematic representations of membrane models as of 1966. (*A*) The Davson-Danielli-Robertson (DDR) model with its lipid bilayer, covered over both surfaces by unfolded protein monolayers. (*B*) The Benson model, consisting of lipoprotein subunits of intertwined fatty acyl and polypeptide chains. The ionic groups of the lipids and proteins are confined to the water surfaces. (*C*) An early version of the lipid-protein mosaic model, with amphipathic integral proteins interspersed with lipid bilayer (see text for details).

THE THERMODYNAMICS OF PROTEIN STRUCTURE

Hydrophobic Interactions

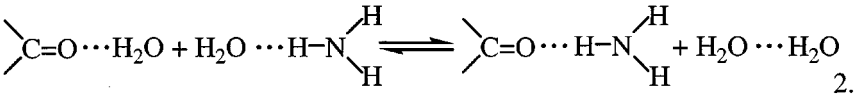
By 1964, considerable advances had been made in understanding the structures of water-soluble protein molecules. It was appreciated that a considerable (thermodynamically favorable) decrease in free energy accompanied the removal of hydrophobic amino acid residues from contact with water into the more hydrophobic milieu of the interior of the protein molecule. (This is the same principle involved in the immiscibility of oil and water, which, despite the massive efforts by owners of ocean-going petroleum tankers to prove to the contrary, still holds true). The simple model system for this hydrophobic effect, the equilibrium (1)

$$CH_4 \text{ (in water)} \rightleftharpoons CH_4 \text{ (in benzene)}, \qquad 1.$$

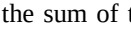
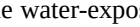
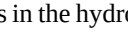
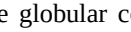
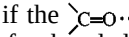
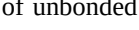
is accompanied by a favorable change in free energy, ΔG, of –2.6 kcal//mol (7). The overall globular structure of most water-soluble proteins provides compacted interior domains where hydrophobic residues are often sequestered away from contact with water, which greatly contributes to the stability (i.e., lowering the free energy) of the entire structure. The first water-soluble proteins to have their entire three-dimensional structures determined to 3 Å resolution by X-ray crystallography, myoglobin (8), and hemoglobin (9), demonstrated this structural principle.

Hydrogen Bonding

Many polar groups in proteins are capable of forming hydrogen bonds, the largest number of these being the peptide C=O and N-H groups. In a water milieu, the equilibrium (2) (where $\cdots$ represents a hydrogen bond)



2.

has a $\Delta G \sim 0$ (10), meaning that the sum of a  hydrogen bond formed in the hydrophobic interior of a globular protein plus the $H_2O \cdots H_2O$ bond formed between the released H_2O molecules, is not energetically favored over the sum of the separate  and  hydrogen bonds formed on the water-exposed surface of the protein molecule. Formation of  bonds in the hydrophobic interior, in this sense, doesn't help to drive the formation of the globular conformation of the protein molecule in water solution. However, if the  bonds do not form in the globular interior, burying each pair of unbonded  and  groups costs about 4 kcal/mol in free energy. Similar considerations apply to other hydrogen-bonding polar groups. In other words, in order to create a stable, lowest free-energy globular conformation, a substantial fraction of the groups capable of forming hydrogen bonds must do so with one another in the globular hydrophobic interior of the protein molecule in the absence of H_2O molecules with which to bond.

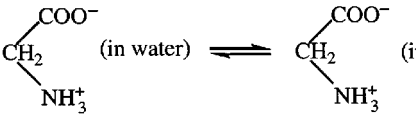
Some water-soluble proteins and many membrane proteins contain covalently attached saccharide residues, which have numerous hydrogen-bonding OH groups as well as ionic residues. By the same reasoning given in this and the following sections, it can be shown (1) that saccharide groups exhibit a lower, more favorable free energy in contact with water than buried in the hydrophobic interior of proteins or membranes.

Hydrophilic Interactions

Several of the amino acids have side chains that are ionized at pH 7: R, K, and H are positively charged, D and E negatively. Can these residues be buried in a thermodynamically favorable, or least unfavorable, free energy in the interior of a water-soluble globular protein away from contact with the water solvent, with its extraordinarily large dielectric constant? Several possible mechanisms to achieve such burial may be considered. Burying an isolated ion in the low dielectric constant medium ($\sim$2) of the hydrophobic interior is ruled out because it would cost a great deal of energy and is entirely unlikely. An ionic group can, however, be buried as a polar, nonionic group by discharging it first. For example, the carboxylate ion of D and E may be discharged by the reaction: $-COO^- + H_3O^+ \rightleftharpoons -COOH + H_2O$. The positive (unfavorable) ΔG for this process is given by 2.303 RT $|(pH - pK)|$, where R is the gas constant, T the absolute temperature, pK is log K, the dissociation constant of the group, and pH is the ambient pH. For a carboxyl group of $pK = 4.5$ to be protonated at pH 7.0, $\Delta G = +3.3$ kcal/mol at 25°C and is about the same for lysine (pK 9.5); it is largest for arginine ($pK \sim 12$), and least for histidine ($pK \sim 7$). Thus burying an ionic residue in the protein interior by first discharging it is, with the exception of histidine, quite unfavorable energetically. [This, however, is not taking into account the favorably

negative ΔG of simultaneously burying the hydrophobic remainder (mostly methylene groups) of each of these amino acid residues in the protein interior.]

In principle, another way of burying an ionic group is to include it as an ion pair (−+) with an oppositely charged group. This has often been proposed, although in 1971 (1) I had already pointed out its prohibitively large free-energy cost. A model reaction for this process is the transfer of the zwitterion glycine from water to a nonpolar, or less polar, solvent



3.

The ΔG for this process may be determined approximately from the equilibrium solubilities of glycine in water and other solvents as RTln (Xw/Xs), where Xw and Xs are the mol fractions of glycine in the saturated solutions in water and the solvent, respectively, at the temperature T. It is a fact (11) that the solubility of glycine decreases sharply with the decreasing dielectric constant, or increasing hydrophobic nature, of the solvent: For acetone (dielectric constant ∼20 and still fully miscible with water), ΔG is already +6 kcal/mol (1). For the globular interior of a protein molecule (dielectric constant ∼2, approximately that of a liquid hydrocarbon), this positive ΔG would be much larger. The conclusion is that burying charged groups as ion pairs in a protein interior away from contact with water is energetically very costly and correspondingly quite improbable. However, this has occasionally been suggested to happen in the interior domain of membrane proteins and is almost certainly incorrect.

THE THERMODYNAMICS OF MEMBRANE STRUCTURE

The foregoing thermodynamic principles developed for water-soluble proteins apply generally to all biological systems in their particular environments. (The DNA molecule is a wonderful example.) These principles have provided the theoretical basis for our investigations of membrane structure in the period 1964–1972.

Membrane Models (1966–1972)

I have already made reference to the DDR model of membrane structure (Figure 1*A*) (6). In 1966, Benson (12) published a model (Figure 1*B*) (the lipoprotein subunit model) in which the protein chains are interspersed with and wound around the fatty acyl chains of the phospholipids in the membrane interior to produce discrete lipoprotein subunits that, packed against one another in the plane of the membrane, constitute the membrane. The lipid is not in the classical bilayer form because it is disrupted by the associated protein chains. The polar head groups of the lipids and the ionic residues of the proteins are located in contact with water on the two membrane surfaces, whereas the hydrophobic residues of the protein

are located largely within the hydrophobic membrane interior, away from contact with water.

Also in 1966 (13), we published an early version of the lipid-protein mosaic model of the membrane (Figure 1*C*), based on the thermodynamics I discussed in the previous section. Similar ideas were put forward independently by Wallach & Zahler (14). The lipids and the (integral) proteins (as we later referred to them) are arranged largely independently of one another in a mosaic pattern in the plane of the membrane.[1] The hydrophobic fatty acyl chains of the lipids and a large fraction of the nonpolar amino acid residues of the integral proteins are sequestered in the membrane interior away from contact with water. The ionic and saccharide groups of the lipids are in direct contact with water. The lipids are organized as a bilayer, interrupted by the intercalated integral proteins. This is consistent with the finding (4) mentioned above that the lipid bilayer constitutes only about one half the red blood cell membrane surface area. I suggested that integral proteins occupy the remaining half. The integral protein may protrude into the water from either one or both of the membrane surfaces; in the latter case, the protein spans the membrane. These extramembranous domains contain all the charged amino acid residues of the protein in contact with water. The integral proteins are therefore proposed to be amphipathic polypeptides, having one or two hydrophilic domains exterior to the membrane, connected to a generally hydrophobic domain that is confined to the interior of the membrane.

How does each of these three models fare in meeting the thermodynamic criteria outlined in the previous section? In the DDR model (Figure 1*A*), the unfolded protein chains on the two membrane surfaces would perforce have most of their hydrophobic amino acid residues exposed to contact with water. The ionic and polar head-groups of the lipids in the bilayer, on the other hand, would be significantly shielded from contact with water by the sheets of unfolded proteins overlying them. Both of these features are thermodynamically quite unfavorable. (In addition, the structural blandness of the DDR model makes it difficult to imagine how a membrane might exhibit a wide range of enzymatic, transport, and transmembrane signaling properties. The DDR model sometimes was pictured with pores through the lipid bilayer, to account for ion permeability, without any justification, theoretical or evidential, in support.)

By stretching out the polypeptide chains and placing them in direct contact with individual hydrocarbon chains of the lipids, the Benson model (Figure 1*B*) would not allow the protein peptide groups $\rangle C{=}O$ and $\rangle N{-}H$ to form many hydrogen bonds with one another; likewise for other polar residues on various amino acid

[1]The recent evidence (cf. 16) for the existence of separate lipid-protein domains within the lipid bilayer (e.g., so-called lipid rafts and caveolae) are not at odds with the lipid-protein mosaic model. I had suggested that the model could accommodate the association of a "fraction of the membrane lipid with the protein moieties, creating a mosaic of [some] lipoproteins interspersed with the remaining lipid in a bilayer" (1, p. 198).

side chains. This would be one of the main factors contributing a large unfavorable free energy to the Benson membrane structure in water. Furthermore, the lipid is not organized as a bilayer, which does not maximize the fatty acid hydrophobic interactions.

The lipid-protein mosaic model (Figure 1*C*) was designed to be thermodynamically feasible. The amphipathic integral proteins have their hydrophobic amino acid residues largely buried in the membrane interior away from contact with water, and their polar, non-ionizable residues are distributed between both exterior and interior domains. Their ionizable residues are essentially all in contact with water on the exterior, extramembranous domains of the integral proteins. The ionic and polar saccharide moieties of the phospholipids are exposed to water on both sides of the bilayer. This structure largely maximizes both hydrophobic and hydrophilic interactions. In the membrane interior, the  and  peptide groups can hydrogen bond to one another (see further below), as can at least some of the side chains of the polar amino acid residues. The wide range of protein to lipid ratios in different membranes mentioned above can easily be accommodated by the lipid-protein mosaic model. (The interesting functional possibilities of the lipid-protein mosaic model were also appreciated, and are discussed below.)

Extensions of the Lipid-Protein Mosaic Model in 1971

In 1970–1971, I had the opportunity of a sabbatical leave to elaborate upon and extend the lipid-protein mosaic model (1), although there had still been no membrane proteins isolated and their structures analyzed. At about that time, however, there were other types of experiments published that were at least consistent with some features of the lipid-protein mosaic model. da Silva & Branton proved in 1970 (16), by freeze-etching experiments in electron microscopy, that protein molecules were deeply embedded in membranes and appeared to be arranged in a mosaic within the lipid bilayer. Bretscher in 1971 (17), using chemical labeling methods, demonstrated that a major protein of the red blood cell membrane was exposed to water on both sides of the membrane (i.e., spanned it). (The Bretscher result is consistent with either the Benson or the lipid-protein mosaic models, but not the DDR.) In 1968, Lenard and I (18) showed that 70% of the phosphorylamine residues of the membrane phosphoglycerides could be removed from intact red blood cell membranes by phospholipase C action, without changing the protein circular dichroism spectrum or the conformational stability of the proteins toward increasing temperature, which are characteristic of the untreated membranes, results that are most consistent with the lipid-protein mosaic model.

INTEGRAL AND PERIPHERAL PROTEINS One extension of the lipid-protein mosaic model (1) proposed that there are two quite different types of proteins associated

with membranes. One type is the membrane-embedded integral protein and the other is a peripheral protein. I anticipated that amphipathic integral proteins would generally be insoluble in water because of their predicted large transmembrane clusters of hydrophobic residues. Certain membrane proteins, however, such as cytochrome c of mitochondrial membranes and spectrin of red blood cell membranes, were exceptions to the rule. These proteins could be isolated intact and without associated lipid by mild treatments of their respective membranes, and when isolated behaved much like ordinary water-soluble proteins. Some investigators viewed these proteins as typical of all membrane proteins generally, but I suggested that these proteins were of another category: peripheral to the membrane, attached noncovalently to specific integral proteins where these protruded from the bilayer into the aqueous phase. The attachment would be via forces that operate to produce specific water-soluble protein aggregates, such as the four polypeptide chain hemoglobin molecule.

Different peripheral proteins were projected at the time to play many diverse and important roles in membrane biology (19): in signal transmission across membranes (see below); in binding extracellular matrices to the exterior face, and cytoskeletal elements to the interior face, of plasma membranes; and in limiting the diffusion of membrane components. This last function includes a role in the formation of distinctive membrane domains such as in initiating endocytic vesicles in membranes, or in synapses at neural and neuromuscular junctions, or in cell adhesion sites. These and many other essential functions have since been proposed and established for particular peripheral proteins of different membranes, but they are not discussed in depth here.

THE DOMINANCE OF THE α-HELIX IN HYDROPHOBIC DOMAINS OF INTEGRAL PROTEINS Because of the energetic desirability, referred to above, of forming the maximum number of $\rangle C{=}O \cdots H{-}N \langle$ peptide hydrogen bonds in the hydrophobic interior domain of the integral protein, and because of the fact that the α-helix is the most efficient structure for the formation of these bonds, I suggested that these interior domains "may all be largely in the α-helical conformation" (1, p. 201), instead of the mixed structure shown in Figure 1*C*. (Peptide hydrogen bonds can also be made between adjacent β-pleated sheets, but where the numbers of adjacent β chains are small (<10), the two outer chains' hydrogen-bond formation is incomplete. However, see Porins, below.) An inference drawn from this conclusion is that in order to traverse the hydrophobic interior of the bilayer ($\sim$35 Å), a single α-helix with each amino acid residue translated about 1.5 Å along the axis of the α-helix would require a stretch of $\sim$23 mainly hydrophobic residues admixed with some polar, but, few, if any, ionic residues, in a continuous sequence. Such long stretches of mainly hydrophobic sequences occur very infrequently in water soluble proteins, and this was suggested to be the critical structural distinction between water-soluble and integral membrane proteins.

DIFFERENT STRUCTURAL TYPES OF INTEGRAL PROTEINS

Monotopic Proteins

I was then also led to suggest that integral proteins might be of several structural types, each with its own functional properties. One type might have its sequence in "two distinct halves [hydrophilic and hydrophobic]. . . that fold up more-or-less independently of each other to give three-dimensional exterior and interior regions to the molecule," as apparently occurs with cytochrome b_5 of microsomes. This integral membrane protein in situ is cleaved by trypsin into two parts, "one carrying the b_5 activity, which is soluble in water, the other remaining membrane-bound [(20)], which confers hydrophobicity on the entire intact molecule and presumably is responsible for its attachment to the membrane" (1, pp. 199–200). This type of integral protein might have a structure like that in Figure 2*A*, incorporating the idea discussed above that the membrane-intercalated hydrophobic domain is likely to be an α-helix. This structure allows for only a single peptide bond cleavage to separate and solubilize the external hydrophilic domain from the membrane-intercalated hydrophobic domain. (This type of hypothetical structure is now well-known, and is referred to as monotopic.)

Polytopic Proteins

On the other hand, "it is conceivable that the single polypeptide chain could have successive segments weaving in and out of the interior and exterior regions of the protein" (1, p. 200), as depicted schematically in Figure 2*B*, again with the several interior hydrophobic domains in the α-helical conformation. "In [such a] case, proteolytic digestion [of the protein in situ in the membrane] would not be expected to cleave off the whole of the exterior regions of the protein" (1, p. 200). (Such hypothetical integral protein structures with varying numbers of hydrophobic transmembrane α-helices are now known to be common and are referred to as polytopic.) Some possible functional differences between monotopic and polytopic integral proteins that were deduced at the time are discussed below.

Where the exterior domains of the amphipathic integral proteins that are in contact with water are of sufficient length, it is likely that they would fold up into a conformation that is more-or-less globular, with the structural characteristics of water-soluble proteins. In the case of the polytopic integral proteins, the several hydrophilic loop regions connecting successive transmembrane helices, together with the one or both N- or C-terminal domains protruding into the aqueous phase from the same side of the membrane, would be expected to associate noncovalently to form a somewhat globular domain on each side of the membrane, each domain connected by several peptide linkages to the opposite ends of the several helices in the transmembrane domain (Figure 2*B*).

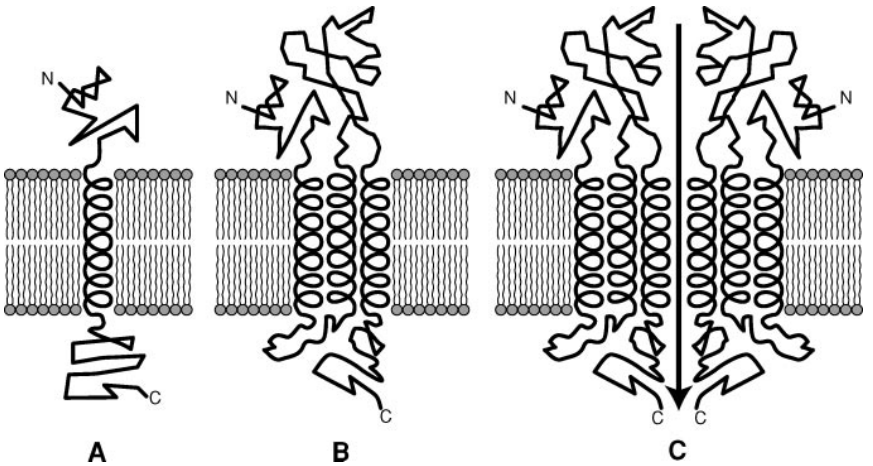


Figure 2 The lipid-protein mosaic model in 1971, with three different proposed types of integral protein structures spanning the membrane: (*A*) A monotopic protein molecule with a single stretch of mostly hydrophobic and non-ionizable amino acids in α–helical conformation within the bilayer, connected by single peptide bonds to two hydrophilic exterior domains extending into the water on both sides of the membrane. (*B*) A polytopic amphipathic integral protein molecule with, in this case, three transmembrane hydrophobic α–helical stretches connected by hydrophilic sequences extending into the two aqueous phases. The number of transmembrane helices and the membrane sidedness of the N and C termini can differ with different polytopic proteins. (*C*) A subunit aggregate of identical or homologous subunits related by a pseudo-axis of symmetry down the water-filled transmembrane channel along the central axis of the channel, serving in the specific transmembrane transport of small hydrophilic molecules and ions. The numbers of subunits in the aggregate, the numbers of transmembrane helices per subunit, and the membrane sidedness of the N and C termini can differ with different transport proteins.

Transport Proteins

Another type of integral protein structure that I proposed in 1971, depicted schematically in cross section in Figure 2*C*, was designed to provide a thermodynamically feasible mechanism whereby small ions and hydrophilic compounds might be transported across membranes. Because hydrophilic species are insoluble in hydrophobic media, such transport cannot occur by simple diffusion across the lipid of the membrane. The "rotating carrier" model of transport was widely considered in the 1960s. In this model, a transport protein is embedded in the membrane by some unspecified means, containing an active site for a specific hydrophilic species to be transported across the membrane, a site that is exposed to the aqueous phase. On receipt of some signal, the transport protein would rotate within and diffuse across the membrane, depositing the hydrophilic species on the opposite side of the membrane. I considered this transport model to be thermodynamically unsatisfactory

because presumably it would require that the ionic and hydrophilic surface domain of the protein move entirely across the hydrophobic lipid bilayer to the other side. Instead, I proposed (1) that all such transport proteins consisted of a small number of specific transmembrane integral protein subunits (generally either identical or homologous to one another so as to form a stable aggregate) that were noncovalently bound to one another around an axis of rotational pseudo-symmetry, creating a continuous water-filled channel along the axis of the aggregate from one side of the membrane to the other (Figure 2*C*). No example of such a subunit aggregate integral membrane protein with a central water-filled channel was known at the time, but such structures were well-known among water-soluble subunit aggregate proteins such as hemoglobin (9). The thermodynamic structural constraints on the model in Figure 2*C* are similar to those on the integral proteins depicted in Figures 2*A*,*B*, except that the amino acid residues of the helices lining the transmembrane channel could occasionally be ionic or hydrophilic if they were in contact with the water in the channel. With an appropriately positioned active site on one of the subunits for a transportable hydrophilic ligand and some kind of gate in the channel (as represented schematically, for example, in Figure 3), a quaternary conformational change requiring only little energy could be induced in the aggregate that would then expose the active site and bound ligand in the channel to the other side of the membrane, where the ligand could be released.

In the ensuing years, many integral membrane proteins involved in transport have been studied chemically, a few have been studied structurally by electron

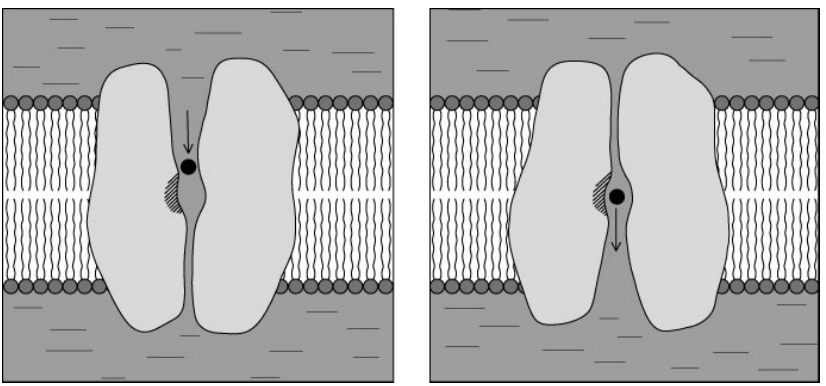
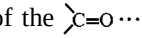


Figure 3 A schematic mechanism of transmembrane transport by a subunit aggregate integral protein with a structure like that in Figure 2*C*. It includes an active site (*shaded*) on a protein subunit that is specific for the small hydrophilic molecule (*dark sphere*). For active transport the aggregate can exist in two conformations, one favored either by the noncovalent binding of an allosteric modulator, a change in the voltage gradient across the membrane, or by covalent bond formation (e.g., by kinase action). This modification causes a quaternary rearrangement (*left panel* to *right panel*) of the subunits that releases the ion to the aqueous compartment on the other side of the membrane.

microscopy and X-ray crystallography, and most have been found to conform in essential features to the subunit-aggregate model of Figure 2*C*, with varying numbers of identical or homologous subunits in different such proteins.

Although most of the transmembrane domains of each of the subunits in such transport proteins were suggested and later found to be composed of α-helices, for the reasons previously given, there is one class of transport proteins called porins that is the exception; their general structure is discussed next.

Porins

We suggested above that the transmembrane domains of integral proteins would essentially all be α-helices because this was the most efficient way of forming the C=O···H–N peptide hydrogen bonds required for the formation of stable polypeptide structures in a lipid bilayer. However, in 1978 Kennedy (21) proposed that a stable membrane-intercalated barrel-like protein structure could be constructed with all of the peptide hydrogen bonds formed, which contained only β-pleated sheets and no α-helices. The requirements were that the β-sequences forming the staves of the barrel were sufficiently long to span the membrane and their numbers sufficiently large ($\sim$12–20). In such a case, a single polypeptide chain containing the $\sim$12–20 successive sequences of the long β-strand-forming stretches might produce a barrel of antiparallel β-strands in which all of the  peptide hydrogen bonds could be formed between adjacent strands because the low degree of curvature of the multi-β-stranded barrel would not induce undue bending strain on the C=O···H–N hydrogen bonds. Every β-strand in the barrel would have successive residues facing the inside (aqueous core) or the outside (lipid bilayer) of the barrel, and the membrane-spanning portion of the β-strand would be restricted to amino acid sequences that accommodated this alternation of hydrophilic and hydrophobic interactions. The water-filled core of the β-barrel might be of significantly larger diameter that that of the water-filled channel of the subunit-aggregate proteins (Figure 2*C*), with each subunit containing only a small number of α-helical subunits. This would generally make the porins capable of transporting larger hydrophilic species than the subunit-aggregate proteins.

Such β-barrel integral proteins were subsequently demonstrated to be characteristic of the transport proteins called porins, by Weiss et al. (22) and Jap (23). The porins apparently are restricted to the outer membranes of gram-negative bacteria and the outer membranes of mitochondria, and are many fewer than the ubiquitous subunit-aggregate type of transport proteins represented in Figure 2*C*.

Diffusion Across the Plane of the Membrane

These considerations of the thermodynamics of transfer across the membrane, besides their relevance to the transport of hydrophilic small molecules and ions, also bear on the general problem of membrane asymmetry. I anticipated that once an integral protein became entirely incorporated in a membrane, with its hydrophilic

domains protruding from the bilayer into the aqueous phase, it was thermodynamically highly unlikely for these domains to be transferred across the membrane during the lifetime of the protein in the membrane. If during integration of the integral protein in the bilayer, it was inserted in a directional manner, this would therefore lead to an asymmetric membrane, one face having a totally different protein domain composition and character from the other.

The asymmetrical distribution of membrane-bound oligosaccharides on the two surfaces of membranes had been demonstrated several times (cf. 24–26) before we did. We developed ferritin-conjugated plant agglutinins as specific electron stains for oligosaccharides and demonstrated their specific asymmetric binding to the exoplasmic surface of several different membranes (27, 28), including the luminal surface of the endoplasmic reticulum (29). Antigenic epitopes on integral membrane proteins were likewise each shown, by specific ferritin–conjugated antibody labeling (30, 31), to be located on hydrophilic domains of integral proteins exclusively on one surface of their respective membranes. Integral proteins do not exist in an equilibrium distribution across the membrane. (This also means that a peripheral protein that binds specifically to one hydrophilic domain of a particular integral protein will also be asymmetrically bound to its associated membrane.)

Similar considerations apply to membrane lipids that have ionic or oligosaccharide head groups. These lipids do not normally transfer across synthetic phospholipid bilayers or resting membranes at any significant rate. However, during certain biochemically active stages of the cell cycle, transfer of some phospholipids that are ionic at pH 7.0, such as phosphatidyl ethanolamine in bacterial inner membranes, are transferred from one half bilayer (where they are synthesized) to the other (32). The mechanisms for such transfers are not understood but appear to involve the mediation of special integral proteins called flippases (cf. 33). The lipids of membranes are nevertheless asymmetrically distributed in the two halves of the bilayer (see below). Whether this distribution is an equilibrium one for each of the lipids (determined, perhaps, by the nonequilibrium absolutely asymmetrical distribution of the integral proteins in the particular membrane) is not clear. If the lipid flippases are either variably present or variably inactive during the cell cycle, an equilibrium distribution of the individual lipids in the two half bilayers may exist only at certain times, but not at others.

Diffusion in the Plane of the Bilayer

The lipid-protein mosaic model, with the integral proteins embedded in the lipid bilayer, raises the question, what is the distribution of a protein in the plane of the membrane? The lipids in many membranes at 37° had already been shown by 1971 (through a variety of physical methods) to be in a fluid rather than a solid state (34–36) if their fatty acid moieties collectively contained sufficient numbers of unsaturated linkages. If a membrane was a lipid-protein mosaic consisting of individual integral proteins dispersed in a fluid lipid matrix, the membrane might in effect be a viscous two-dimensional liquid-like solution. In a seminar that I gave at the Rockefeller Institute in 1971, I raised the issue of diffusion of proteins in the

plane of the membrane, and Siamon Gordon informed me of the paper that had just been published by Frye & Edidin (37). In this remarkable paper, the authors produced mouse-human cell fusion heterokaryons and followed, with time after cell fusion, the surface distributions of a mouse and a human cell surface protein antigen using the double immunofluorescent technique. Immediately after cell fusion the two antigens were confined to the two distinct halves of the heterokaryon surface, but after about 40 min at 37°, the two antigens were completely intermixed over the entire heterokaryon surface. With suitable controls, the authors concluded that global free diffusion of the two antigens in the membrane had occurred.

In our own experiments around this time, we had shown by immunoelectron microscopy that the Rho (D) antigen on human red blood cell membranes (30) and the H-2 alloantigen on mouse red blood cell membranes (31) appeared to exist in small clusters that were randomly distributed over the membrane surfaces. In the light of the Frye-Edidin experiment, these results were consistent with a limited amount of free diffusion of these proteins to form the clusters in the membrane, but we could not rule out the possibility that the clusters were stable aggregates in the membrane. As it turned out, the adult human red blood cell is unique in limiting membrane diffusion because integral proteins in its membrane are bound to an underlying continuous matrix (skeleton) of peripheral proteins, including spectrin, that severely restricts the lateral diffusion of integral proteins in the membrane (cf. 38). Our work employed red blood cells exclusively at that time, and so we missed observing the global diffusion of membrane proteins in our experiments that Frye & Edidin observed with lymphocytes. Other experiments carried out at about the same time by Taylor et al. (39) on the antibody-induced clustering of a cell surface antigen of lymphocytes (the so-called capping phenomenon), cells that do not have a continuous membrane skeleton, also attested to the global character of the movement of protein antigens in membranes. With the appearance of the Frye-Edidin paper, we incorporated the concept of global diffusion into our lipid-protein membrane model, which appeared as the fluid mosaic model in February 1972. Our three-dimensional representation (figure 3 in Reference 1) of the lipid-protein mosaic model, unchanged, was now an instantaneous snapshot of the fluid mosaic model.

The Primary Structure of Glycophorin

In 1978, Marchesi and his colleagues (40) reported the complete amino acid sequence of a major glycoprotein of the human red blood cell membrane, glycophorin A. This was the first complete sequence of an integral membrane protein to be published. The work was the culmination of a difficult experimental program of protein isolation, purification, and peptide (especially of glycopeptide) sequence analysis, before the days when protein sequences were much more readily obtained from the corresponding cDNA sequence.

Glycophorin A is 131 amino acids long, recognizably divided into three sequential domains: with an N-terminal sequence of 72 amino acids, containing 23 ionic residues and the positively charged N terminus, and all of the large number

of saccharide units of the protein; a succeeding stretch of 23 residues, mostly hydrophobic, together with a few polar but not a single ionizable residue; followed by a final sequence of 36 residues containing 12 ionic residues plus the negatively charged C terminus. This structure perfectly fits the prediction of the structure of a monotopic integral protein (Figure 2*A*) of the plasma membrane, with an exterior hydrophilic domain containing many ionic residues and all of the oligosaccharides of the molecule (28), followed by a bilayer-spanning hydrophobic domain containing 23 amino acids (presumably in an α-helix) but no ionizable residues, followed in turn by a cytoplasmic hydrophilic domain containing many ionic residues but no oligosaccharide. The N terminus in this case faces the exoplasm, the C terminus the cytoplasm.

SOME FUNCTIONS OF MEMBRANES PREDICTED FROM THE FLUID MOSAIC MODEL

Up to this point, we have mainly discussed the structure of membranes, as the subject progressed from 1964 to 1972, leading us to formulate on a thermodynamic basis the fluid mosaic model of membrane structure. I turn now to our proposals for the mechanisms of a number of membrane functions that emerged from further consideration of this model.

Signal Transmission Across Cell Membranes

We realized that the presence of amphipathic integral proteins that spanned the plasma membrane of a cell constituted a means of transmitting chemical information (signaling) from the exterior medium into the cell, a subject that was in its infancy at the time. These signaling mechanisms could be initiated by the noncovalent specific transient binding of a water-soluble ligand molecule, say, a hormone (either small, or as large as a protein like insulin) to a site on the exterior-facing hydrophilic domain of a particular amphipathic integral protein. The sequellae to this binding would depend in part on the structure (Figure 2) of that integral protein. (Such protein hormone ligands, by the way, can be construed as one type of peripheral proteins.) The possible effects of such ligand binding to monotopic integral proteins (receptors) is considered first.

Monotopic Proteins

Monotopic proteins (Figure 2*A*) have a single peptide bond connecting the exterior hydrophilic domain to the α–helical hydrophobic domain, followed by another single bond to the interior hydrophilic domain. In the absence of stable aggregate formation by this monotopic protein with itself or other proteins in the membrane, I considered this structure to make it unlikely that any ligand-binding conformational change induced in the external domain would be directly transmitted across the single helical domain to the interior-facing hydrophilic domain of the

same integral protein molecule. Instead, the fluid mosaic model suggested the following mechanistic variation on the Monod-Changeaux-Wyman model of protein cooperative phenomena (41). The exterior hydrophilic domain of the monotopic protein "can exist in one of two conformational states, one of which is favored by [specific] ligand-binding. In the normal unbound conformation, the integral protein is monomolecularly dispersed within the membrane, but in the conformation promoted by ligand binding, its aggregation is thermodynamically favored. The binding of a ligand molecule at one integral protein site, followed by diffusion of [a] nonliganded [molecule of the] protein to it, might then lead to [their] aggregation and spontaneous change in conformation of the [aggregate-bound nonliganded molecule]" (2, p. 729). The induced proximity of the two interior hydrophilic domains of the now dimerized receptor may then activate them. This predicted mechanism of diffusion-controlled cooperative conformational changes has since been found to explain the activation of many monotopic membrane receptors, such as the majority of monotopic receptor tyrosine kinases, where the binding of a hormone to the exterior hydrophilic domain results in a dimerization of the receptor, followed by a mutual tyrosine phosphorylation within their now dimerized and activated interior hydrophilic domains (cf. 42).

[More generally, I suggested that the newly discovered global diffusion in membranes had as its "real purpose. . .to permit some critical integral proteins to retain their translational mobility in the plane of the membrane, as an obligatory step in their function" (2, p. 730). This has since become clear, for example, in the formation of intracellular synapses and other adhesions, in the endocytosis of membrane proteins, and in intracellular signaling.]

Polytopic Proteins

With polytopic integral proteins (Figure 2*b*), the situation is quite different. The polytopic protein molecule can be a single integrated structure, unlike the monotopic molecule, which contains three structurally non-integrated domains. The exterior-facing hydrophilic domains and separately, the interior-facing hydrophilic domains are most likely to be structurally integrated at their surfaces with their neighboring hydrophobic helical sequences such that the binding of a specific ligand to the exterior facing domain can lead to a conformational change that affects the structure of the entire protein molecule in the membrane, including the interior-facing domain. The signal could thus be transmitted directly across the single polytopic molecule, and not require the prior diffusion and aggregation of the ligand-bound integral protein in the membrane. This appears to be the general mechanism for transmembrane signaling involving polytopic proteins (Figure 2*B*), such as with the superfamily of heterotrimeric G protein–coupled receptors (GPCR) (see 43). The noncovalent binding of a ligand (except for the case of the rhodopsins, where the retinal ligand is covalently bound) to the exterior hydrophilic domain of this seven-transmembrane helical protein receptor triggers a conformational change that apparently affects the structure of its integrated interior hydrophilic

domain so as to change its binding characteristics for the components of the heterotrimeric G protein in the cytoplasm. This initiates one of several intracellular reaction cascades depending upon the particular G protein involved (44).

Transport Proteins

At a time when no thermodynamically satisfactory model of a transport protein was available and no such protein had as yet been isolated and characterized, I was the first to propose (1) a thermodynamically feasible structure and mechanism for them, namely, that transport proteins would generally consist of small aggregates of identical or homologous transmembrane amphipathic subunits forming a water-filled transmembrane channel down the central axis of the aggregate (Figure 2*C*). Transmembrane transport of specific ions and hydrophilic small molecules would occur by their movement through the appropriately structured water channel characteristic of each specific transport protein aggregate (Figure 3), via an appropriately stimulated conformational change in the channel. I applied these ideas to propose a mechanism for the ligand-gated ion transport protein, the acetylcholine receptor of the neuromuscular junction.

It was known by 1970 that acetylcholine binding to the acetylcholine receptor could affect the ion permeability of the membrane, but the receptor had not yet been characterized in any detail, and the activation process was not understood. I suggested in 1971 that "the binding of acetylcholine at a specific receptor site on one subunit [of the subunit aggregate constituting the receptor] could induce a quaternary rearrangement of the different subunits of the same protein molecule and thus change the ion-permeability characteristics of a pore extending down the central axis of the aggregate" (1, p. 206–7). This predicted model of the structure and function of the acetylcholine receptor has since been verified in its essential details (45–47). The receptor consists of five subunits: two identical α, and one each of β, γ, and δ subunits, each homologous in amino acid sequence to the α protein (45). The aggregate, of five-fold rotational pseudo-symmetry, has a transmembrane water-filled channel down its central axis (46) which, upon the binding of acetylcholine to the exterior domain of one of the two α subunits (45), causes the channel to undergo a conformational change (47) that increases its ion permeability. In other cases examined in recent years, such as a variety of K^+, Na^+, and Ca^{2+} voltage–gated ion transport proteins, all of which consist of four subunit aggregates, there is a more elaborate structural mechanism for opening and closing the ion channel of the subunit aggregate as determined by high-resolution X-ray crystallographic analysis (cf. 48).

Membrane Asymmetry

The asymmetry of membranes and the associated differences of protein and lipid structures in their two half layers suggested to us some novel properties and functions of membranes. I discuss briefly several of these that occurred to us during the period 1971–1974.

ON THE MEMBRANE INTERCALATION OF INTEGRAL PROTEINS I suggested that the totally asymmetric orientation of the individual kinds of integral proteins across a membrane entailed certain consequences for the process of the initial intercalation of the integral protein molecule into the membrane. I considered that the amphipathic structure of integral proteins would render them insoluble in water solution (chaperones were unknown at the time). "A ribosome with bound messenger RNA may become attached to a cell membrane. There would have to be specificity to this attachment, so that the membrane proteins would ultimately become associated with the right membrane. . . . As the individual protein molecules are made, they would then be inserted directly into the membrane without being solubilized" (1, p. 215). This would account for a directionality to the integral protein insertion process, starting from the N terminus. These speculations preceded the discoveries of the signal peptides and signal recognition particles (cf. 49) but were generally on the right track.

THE BILAYER COUPLE HYPOTHESIS The asymmetric distribution of the different lipids in the two halves of the lipid bilayer was first demonstrated for the red blood cell membrane (50, 51), but it is now known to be a general property of cell membranes. Of the major phospholipids of the red blood cell membrane, phosphatidyl choline and sphingomyelin are concentrated in the exterior half of the bilayer and phosphatidyl ethanolamine and phosphatidyl serine in the cytoplasmic half. The first three of these have zwitterionic head groups, the last has a net negativity charged head group. This means that the surface of the cytoplasmic half layer carries not only a chemically different structure but also a net negative charge from its lipids compared with that of the surface of the exterior half layer. We anticipated that these lipid half-layer differences (ignoring differences owing to the asymmetric distribution of different integral proteins in the membrane), could confer somewhat different properties on the two half layers and differential responses to various perturbations of the lipid bilayer of the membrane. By analogy to a bimetallic couple, we referred to this as the bilayer couple hypothesis (52).

Drug-erythrocyte interactions As one consequence of the bilayer couple hypothesis, we proposed in 1974 (52) that amphipathic drugs, containing both a lipid-soluble hydrophobic domain and an ionically charged group, would distribute differentially into the two half layers of the membrane lipid bilayer of red blood cells, depending on the nature of their ion charge. This would result in different changes in the curvature of the membrane (Figure 4). The normal shape of the red blood cell is the well-known smooth-surfaced biconcave disc (Figure 5*A*). If a positively charged drug such as chlorpromazine (Figure 6), which can be discharged by the removal of its N-bound proton, is added to the cell, it first enters the exterior half layer of the bilayer by its hydrophobic domain, but it can diffuse across the bilayer as the uncharged species, and then reacquire its proton and positive charge when exposed to the water on the cytoplasmic side. There it is electrically attracted to and concentrated in the more negatively charged cytoplasmic

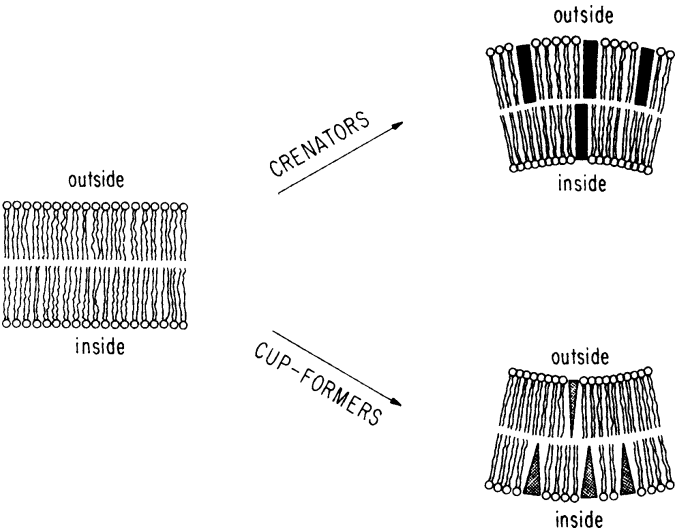


Figure 4 The mechanism of interaction of amphipathic drugs with the asymmetric halves of the lipid bilayer, according to the bilayer couple hypothesis. (See text and Figure 5) (From Reference 52. Copyright 1974, with permission from AAAS.)

half layer, where as a consequence of expanding the area of the cytoplasmic half layer compared with the exoplasmic half (Figure 4), it induces cup–shapes in the intact erythrocyte (Figure 5*C*). On the other hand, the very similar drug methochlorpromazine (Figure 6), because of its permanent positive charge, cannot readily diffuse across the bilayer. Therefore, it concentrates in the exterior half layer, expanding its area relative to the cytoplasmic half layer, thereby producing a crenated-shaped cell (Figure 4; Figure 5*B*). Negatively charged drugs such as

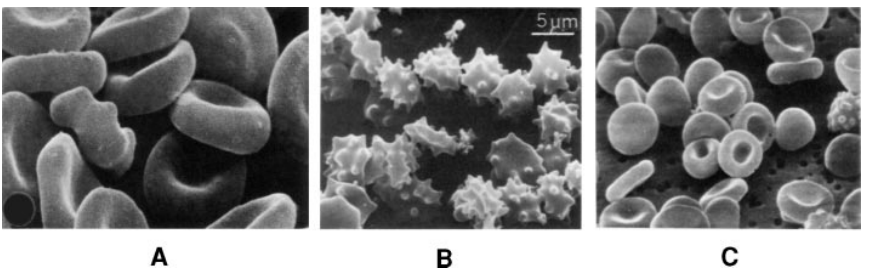


Figure 5 Intact human red blood cells as observed by scanning electron microscopy. (*A*) normal cells; (*B*) crenated cells formed after the addition of 0.2 mm methochlorpromazine (Figure 6); (*C*) cup-shaped cells formed after the addition of 6 μm chlorpromazine (Figure 6). The magnification of Figure 5*A* is slightly larger than for *B* and *C*. See text for details. (From Reference 52. Copyright 1974, with permission from AAAS.)

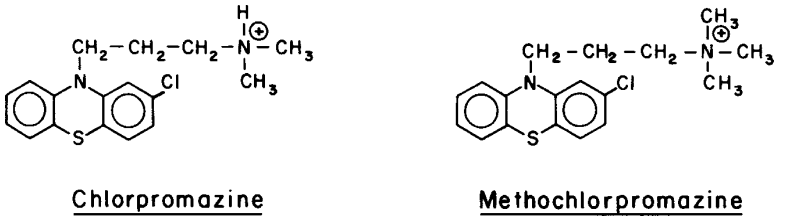


Figure 6 The structures of two structurally similar amphipathic drugs used in the experiments shown in Figure 5.

2, 4-dinitrophenol do likewise (52, 53) owing to electrical repulsion from the net negatively charged lipids in the cytoplasmic half of the bilayer.

Membrane curvature The bilayer couple hypothesis is relevant to the general property of membrane curvature, a subject that has not received the attention it deserves. Many intracellular membranes of eukaryotic cells, e.g., the Golgi saccules, exhibit regions of sharp curvature at their rims that are continuous with their relatively flat domains. Similarly, regions of flat membranes that become vesiculated, as in endocytosis, or vesicles that undergo fusion with flat cell membranes, as in secretion, undergo changes in curvature. At these regions of high curvature, the half layer on the convex side occupies a larger area than its associated concave half layer, and these changes require some specific mechanism to produce. Such mechanisms are generally unknown. Factors that might be involved include the insertion or diffusion and concentration into the regions of curvature of asymmetrically shaped lipids (54); an asymmetrically distributed addition of electric charge on particular lipids (e.g., by phosphorylation); the concentration of asymmetrically shaped or asymmetrically charged membrane-spanning proteins; or the asymmetrical and localized attachment of peripheral proteins such as clathrin (55).

HIGH-RESOLUTION X-RAY CRYSTALLOGRAPHIC STRUCTURES OF THE PHOTOSYNTHETIC REACTION CENTER AND OF RHODOPSIN

This article has concentrated mostly on the early period in our study of the structure of biological membranes, particularly from 1964 to 1974, culminating in the development and exploration of the fluid mosaic model of membrane structure. At the time, the relevant experimental information, especially about the membrane proteins, was not yet available, and the model was based largely on thermodynamic theoretical considerations. Since that early period, membrane structure studies have become an industry. Many integral proteins have been isolated and characterized biochemically and physico-chemically, and the fluid mosaic model has generally been confirmed by such experiments. The most remarkably detailed information

has come from X-ray crystallographic studies at 3 Å resolution, or better, of a number of single integral membrane proteins or protein complexes isolated from their native membranes and crystallized with the use of nonionic detergents. For the purposes of this article, I refer to only two of these studies; one on the photoreaction center (PRC) of photosynthetic bacterial membranes (56), published in 1985; and the other, on bovine rhodopsin (57, 58), published in 2000 and 2002, to determine whether and how well the predictions of the original fluid mosaic model, made 13 to 20 years earlier, fit these remarkably detailed structural results.

The PRC of *Rhodopseudomonas viridis*

The PRC was the first integral membrane protein to have its complete three-dimensional structure determined to better than 3 Å resolution by X-ray crystallography. PRC is a protein complex in the membranes of vesicles inside *R. viridis* cells that acts like a solar battery: It converts light energy into electron energy, and by quantum mechanical electron tunneling between the several kinds of pigments positioned at successive fixed sites within the protein complex, transfers the electrons across the membrane (59, 60). The three-dimensional structure of the PRC membrane complex is represented parallel to the bilayer in the top portion of Figure 7. The complex contains four noncovalently linked proteins: three, H, L, and M, that are transmembrane integral proteins and one, a cytochrome b_5 (Figure 7), that is a peripheral protein attached to the H-L-M complex.

Regarding the predictions of the original fluid mosaic model, they are largely met by the PRC structure. (*a*) The PRC exhibits both monotopic and polytopic types of transmembrane integral proteins, as well as a peripheral protein that is entirely located in the aqueous phase and is attached to the surface of the integral protein complex where the latter protrudes into the periplasmic aqueous phase. (*b*) The transmembrane domains of the integral proteins are entirely α-helical, and range from about 20 to 25 residues in length, as anticipated. (*c*) Most remarkably, of the more than 150 ionic amino acids of the PRC complex, none is located within the 11 transmembrane α–helices except for a few that are situated near either end of a helix where these residues can be in at least partial contact with the aqueous phase. This is in striking accord with the earlier discussion about hydrophobic and hydrophilic interactions.

A closer look at this cross section through the PRC, together with the view from one face of the complex (Figure 7, *bottom*) down through the transmembrane domain, reveals additional interesting features of the structure. (*a*) The homologous M and L chains are related by a pseudo twofold axis of rotation perpendicular to the membrane, but the single transmembrane helix of the H chain is not situated on this axis (Figure 7, *bottom*), as one might initially have expected. This off-axis shift of the H helix might be influenced by its interactions with the large external domain of the H chain and the peripheral cytochrome molecule, both of which are monomeric, and therefore asymmetrically associated with the L-M pseudosymmetric dimer. (*b*) Most of the L and M helices are only rather loosely

packed into an overall oval configuration (Figure 7, *bottom*), with the A and D helices of both chains fairly isolated from the others. (*c*) The five helices of either the L or M chains are not arranged precisely in the same order as in the amino acid sequence, with the E and D helices inverted in their positions.

Bovine Rhodopsin

Rhodopsins are integral membrane proteins of rod and cone cells in the retina of the eye that are centrally involved in animal vision. Rhodopsin is a member of a superfamily of heterotrimeric G protein–coupled receptors (GPCR) (cf. 43), each responding to stimulation by a different specific ligand, generally binding to the receptor at its exoplasmic domain. This activates its particular G protein bound at the receptor's cytoplasmic domain, by a conformational change that is transmitted through the transmembrane domain of the polytopic receptor. All the GPCRs so far studied have seven hydrophobic transmembrane helices. Rhodopsin is unusual in having its ligand, 11-*cis* retinal, covalently bound to the receptor. This bond is within the transmembrane domain of rhodopsin: A Schiff base is formed by retinal with K296 of helix VII (61, 62). With all other GPCRs, the ligand is a water-soluble molecule that binds noncovalently to the exoplasmic or transmembrane domain of the receptor. With rhodopsin, the activation occurs by the absorption of a light photon by retinal, which converts the 11-*cis* form to the 11-*trans* isomer, thereby triggering a conformational change in the transmembrane domain of rhodopsin that is transmitted to its cytoplasmic domain.

Recently, nearly the entire three-dimensional structure of bovine rhodopsin has been determined at 2.8 Å resolution (57, 58) (Figure 8). Some unusual features of this structure, not exhibited by the PRC complex, are of general interest. (*a*) The transmembrane domain has seven generally hydrophobic α-helices, but these helices are usually quite kinked (and therefore locally non-α-helical), often found at proline residues. (*b*) The seven helices are intertwined with one another in a complex structure in an arrangement that is largely unrelated to their order in the amino acid sequence of rhodopsin. Furthermore, the arrangement of the helices with respect to one another differs at different levels in the membrane, which is probably at least partly associated with the complex pattern of the kinking of the individual helices. (*c*) The transmembrane helices are tightly packed into a complex oval array, as seen from the cytoplasmic surface (Figure 8*B*) and the exoplasmic (intradiscal) surface (Figure 8*C*) of the membrane. This is in contrast to the looser aggregation of the PCR helices (Figure 7, *bottom*). (*d*) There is a total of 11 ionizable residues distributed among five of the seven transmembrane helices of rhodopsin. Six of these residues are near the membrane surfaces, probably in partial contact with the aqueous medium, and therefore present no thermodynamic problem. Of the remaining five, D83 is near the middle of helix II; E113 and E122 in helix III, H211 in helix V, and K296 in helix VII (which makes the Schiff

base bond with retinal) are also internal. (*e*) Particularly interesting is the presence of seven individual water molecules at fixed positions within the transmembrane domain, which have to be stably bound in the structure to be detected by X-ray crystallography (58).

Because the notion that amphipathic integral transmembrane proteins even existed, and further that their transmembrane domains were composed largely of hydrophobic α-helices, were largely conjectural in 1971, I had neither the insight nor the courage to think about internally bound water molecules, helical kinking, or possible interhelix arrangements at the time, but they have now become very interesting problems. The rhodopsin structure may have features common to many polytopic integral proteins, compared with the simpler PCR structure, particularly for those proteins, which, like rhodopsin, have to undergo an extended conformational change as part of their biochemical function.

What about the few ionizable residues referred to above that appear to be located in the interior of the transmembrane domain of rhodopsin? As discussed in a previous section, such residues are more stably located if they are in the discharged state in the water-free hydrophobic interior. But some residues such as D-83 and E-113 appear to be hydrogen-bonded to one of the fixed water molecules in the transmembrane domain of rhodopsin. This may further stabilize these discharged ionizable residues in the generally hydrophobic interior. Making and breaking these relatively weak hydrogen bonds may also play a role in any conformational changes involving the membrane-interior portions of the protein.

The complex configurations of the kinked transmembrane helical domains of polytopic integral proteins such as rhodopsin may have important consequences. For example, they may produce crevices in the water-exposed surfaces of such proteins and thereby allow solvent water (and not only the stably bound water molecules as discussed above) to penetrate to some depth into portions of the interior of the transmembrane protein in one or more of its conformations. This transient interior water may make it thermodynamically favorable for certain membrane-interior ionizable residues to ionize transiently in those conformations that allow these residues to be in contact with the water. The crystal structure of rhodopsin shows E113 to be close to the Schiff base linkage, and the existence of an ion-pair between the carboxylate negative charge of the (presumed to be) ionized E-113 and the (presumed to be) positively charged Schiff base has been suggested. As we have demonstrated, however, an ion-pair has a very high free energy (i.e., is highly unfavorable) in a nonpolar environment. These ionizable groups may therefore be ionized only in those conformations that allow water into interior parts of the transmembrane domain. In other conformations in the activation cycle, the region around E-113 and the Schiff base may be closed to water permeation and be hydrophobic. In such a conformation, both ionizable groups would then likely exist in the discharged state and be hydrogen bonded to one another.

Such water penetration into transmembrane crevices could also be involved in the mysterious proteolysis of peptide bonds that seem to be located at some depth in the apparently hydrophobic interior of a polytopic protein (cf. 63). In any event, water must have access to such apparently buried proteolyzable peptide bonds, because the elements of water have to be added across the broken bond. But such crevices might also transiently admit some appropriately shaped hydrophilic domains of proteases to hydrolyze such interior peptide bonds.

Lastly, the proximity of E113 (on helix III) to the Schiff base attached to K296 (on helix VII) of rhodopsin also provides an opportunity to emphasize the obvious fact, too often ignored, that the linear, sequence-ordered topography of the hydrophobic domains of integral proteins that we usually draw from hydropathy plots (64) of polytopic integral proteins has no bearing on the real three-dimensional appositions of the helices in the transmembrane domain.

CONCLUSION

The early days of membrane molecular biology have been treated here from a personal perspective. In the absence of even one isolated and structurally characterized membrane protein, the analysis of membrane structure that I engaged in was mainly of thermodynamic nature. This, together with some critical experiments by others on the global diffusion of proteins in membranes, led me to the fluid mosaic model of membrane structure, which is still valid today as a general overview of the structures and functions of biological membranes. It has held up to the subsequent structural information obtained with many isolated membrane proteins and their complexes. A particularly exciting period in membrane molecular biology has been introduced by the successful application of X-ray crystallographic methods to crystals of individual membrane proteins and protein complexes in nonionic detergents, with a resolution of 3 Å or better. However, it is still a high art to produce usable crystals of such water-insoluble proteins. But the extraordinary insights provided by this method are well worth the effort. Appropriate chemical modification and bioengineering of specific membrane proteins may be useful in making suitable crystals of these proteins easier to produce; for example, methods may be developed such as attaching readily crystallized and already structurally analyzed water-soluble proteins to the membrane proteins in such defined ways that do not affect the structures of either. These and other technological advances can be anticipated in the future and will expedite the coming revolution in the detailed understanding of membrane molecular biology.

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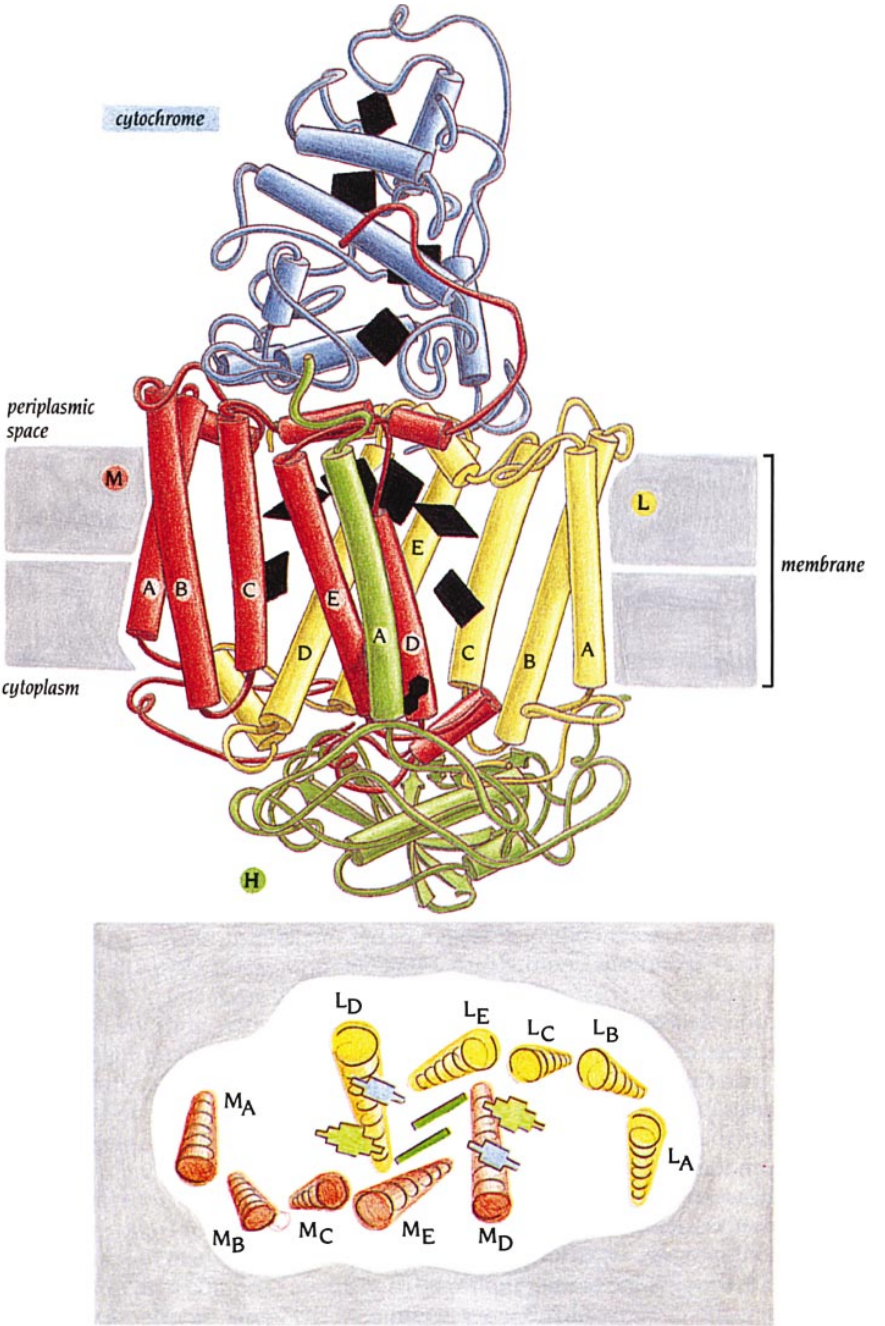

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Figure 7 Schematic views of the X-ray structure of PRC viewed parallel to the membrane (*top*), and only its transmembrane domain viewed perpendicularly to the membrane from one face (*bottom*). Shown are the homologous L (*yellow*) and M (*red*) integral protein molecules with their respective five transmembrane α-helices (in the order A, B, C, D, and E in the amino acid sequence); the H integral protein molecule (*green*) with its single transmembrane α-helix and large hydrophilic cytoplasmic domain, and the peripheral protein cytochrome (*purple*), along with the more or less linear arrangement of the electron transfer pigments and heme groups (*top, black*). (Taken from Reference 56, with permission).

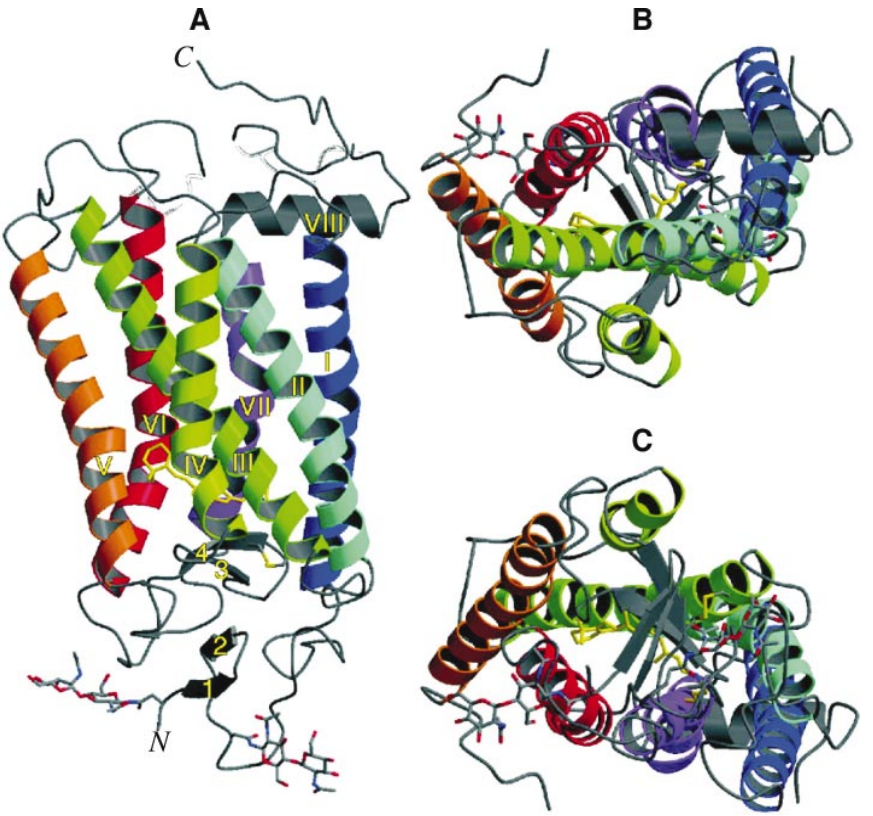

Figure 8 Schematic views of the X-ray structure of bovine rhodopsin: (*A*) Parallel to the membrane, (*B*) view into the plane of the membrane from the cytoplasmic side, and (*C*) from the exoplasmic side of the disc membrane. The seven hydrophobic transmembrane helices are labeled I through VII according to their order in the amino acid sequence. The outer surfaces of the helices in (*A*) coincide, more or less, with the outer surface of the surrounding membrane bilayer. *N* and *C* in part (*A*) represent the N and C termini of the rhodopsin molecule on its exoplasmic and cytoplasmic sides, respectively. Several of the bound pigment molecules are labeled 1–4. (From Reference 63. Copyright 2000, with permission from Routledge/Taylor & Francis Books, Inc.)

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CONTENTS