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Pivotal Ideas

The Structure and Function of Membranes—A Personal Memoir

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Introduction

It is now 30 years since we made our first brief foray into membrane biology (Singer, 1962) and 20 years since the fluid mosaic model was published (Singer & Nicolson, 1972). It seems an appropriate time to look back at how these and other related ideas were generated and what has become of them. This will be a personal view, and is therefore by no means intended to be a full history of recent membrane biology. On the other hand, it may serve as a partial antidote to the versions of the subject that appear in some current textbooks of molecular cell biology, where the logic of the historical development is entirely sacrificed to an orderly arrangement of subject matter as reconstructed through hindsight. This memoir deals mainly with ideas. We have suggested elsewhere (Singer, 1992*a*) that ideas are the poetry of science. Society has come a long way since the Middle Ages, when the most prolific poet was named *Anon.* Nowadays, poets like to have their own names associated with their poems, likewise scientists with their ideas.

Thermodynamics and Membrane Protein Structure

It is difficult for recent generations of scientists to realize the situation that membrane biology was in during the 1950's and 1960's. The subject was mainly of interest to physiologists concerned with ion and metabolite transport through membranes and biochemists concerned with mitochondrial electron transport and oxidative phosphorylation. Relatively little research was addressed to membrane structure *per se,* and membrane proteins were mostly intractable to detailed study. This was before the introduction and widespread use of SDS-polyacrylamide gel electrophoresis for the study of membrane proteins (Shapiro, Viñuela & Maizel, 1967). The generally accepted model for the molecular organization of membranes was the Davson-Danielli-Robertson model (Robertson, 1964) (Fig. 1*A*), which pictured the structure as a railroad-track assembly with a phospholipid bilayer sandwiched between two layers of unfolded protein sheets.

In the 1950's, my activities as a protein physical chemist in the Department of Chemistry of Yale University included studies of the solubility and structural characteristics of water-soluble proteins in nonaqueous solvents. The Doty laboratory at Harvard had been busy studying the structural properties of synthetic polypeptides in nonaqueous solvents, but ordinary proteins were generally thought to be insoluble in media other than aqueous. This turned out not to be true in a significant number of cases (Rees & Singer, 1956). We explored this problem in some detail, and this work led to the review in 1962. The results that we and others had obtained indicated that the conformations of protein molecules very much depended on the nature of the solvent. Although we had not experimented with membrane proteins and knew very little about membranes at the time, as almost an aside we speculated that because "the cellular environment of many proteins contains high concentrations of lipid components in a wide variety of cellular membranes, the gross conformations of these proteins *in situ* may be determined by this association with a nonaqueous environment." This notion set off a train of ideas and experiments that eventually led us to the fluid mosaic model.

The landmark article by Kauzmann (1959) introduced protein chemists to the concept of hydrophobic interactions and the crucial role they play in the thermodynamics of protein structure. Applying these ideas to membrane proteins, we realized that the Davson-Danielli-Robertson model was unsatis-

Key Words fluid mosaic model · integral and peripheral proteins · transport · thermodynamics

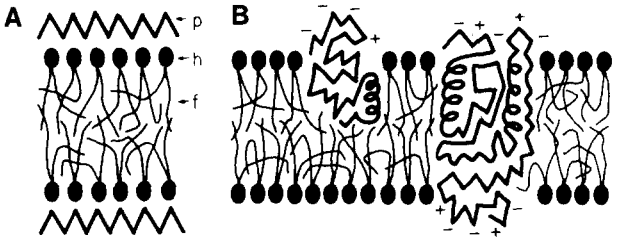

Fig. 1. Schematic representations of membrane structure: (*A*) the Davson-Danielli-Robertson model (Robertson, 1964) and (*B*) the lipid protein mosaic model (Lenard & Singer, 1966). In the former model, the membrane protein (*p*) is organized as two monolayers on either side of the lipid bilayer, with the fatty acyl tails (*f*) and polar head groups (*h*) of the phospholipids indicated. (Modified from Singer, 1971.)

factory thermodynamically in part because it would require many of the hydrophobic amino acid residues of the unfolded proteins to be in contact with water. We therefore suggested (Lenard & Singer, 1966) instead that the molecules of membrane-associated proteins were globular and amphipathic (Fig. 1*B*), with a hydrophobic domain embedded in the hydrophobic interior of the lipid bilayer, and with either one or two hydrophilic domains protruding from the bilayer into the aqueous surroundings. Others (Wallach & Zahler, 1966) presented similar ideas. Subsequently, chemical labeling experiments (Bretscher, 1971), and a detailed analysis of freeze-fracture electron microscopic studies (Pinto da Silva & Branton, 1970), provided the first firm experimental evidence that the molecules of at least some membrane-associated proteins indeed did span the membrane.

The opportunity provided by a timely sabbatical leave allowed me to put together a detailed analysis of membrane structure and the thermodynamics of membrane proteins, which was presented in 1971 (Singer, 1971). This was at a time when there was still no information about the sequence or structure of any membrane protein. The free energies required to embed ionic amino acid residues in a medium of low dielectric constant were estimated and were shown to be quite unfavorable. For example, data from simple model systems showed that burying ionic residues as oppositely charged ion-pairs in a hydrophobic medium is very costly in free energy, contrary to intuition. This is a thermodynamic fact that has often been ignored, and many investigators have continued to insert ion pairs in the interior of membranes in speculative models of different membrane protein structures. In science as in politics, however, ignorance of the law(s of thermodynamics) is no excuse. We suggested that the energetically least costly way to bury an ionic residue is to discharge it first, which takes the least energy for histidine and the most for arginine. These calculations provided some quantitative basis for the prediction that the embedded interior domains of membrane proteins would be largely devoid of ionic residues.

Thermodynamic considerations also led to the prediction that the embedded hydrophobic sequences of membrane proteins "may all be largely in the α-helical conformation" (Singer, 1971, p. 201), because the α-helix is the structure which most efficiently allows interpeptide hydrogen bonds to form. We argued that such hydrogen bond formation must be maximized in order to lower the free energy of the embedded domain. [An alternative way to accomplish this is by the formation of a β-stranded barrel (Kennedy, 1978); *see* the section on Bacterial Porins below].

We envisioned two possible kinds of domain structures. In one case, the exterior hydrophilic and interior hydrophobic domains might be conformationally independent of one another, perhaps linked by a hinge region as had been found for the domains within immunoglobulin polypeptide chains; in the second case, "the single polypeptide chain could have successive segments weaving in and out of the interior and exterior regions of the protein" (Singer, 1971, p. 200). In the latter case, in contrast to the former, the exterior hydrophilic domains and the interior hydrophobic domain would be conformationally interdependent. These suggestions about two different kinds of domain structures have since been verified, the first kind corresponding to Types I and II integral proteins, and the second kind to Type III (Fig. 2). The functional significance of these structural differences is considered later on. Type IV proteins (Fig. 2), whose distinctive characteristic is to form aqueous channels across a membrane, are discussed below.

The Bacterial Photosynthetic Reaction Center (PRC)

These ideas and predictions about membrane structure that were developed by 1972 have largely been borne out by the experimental evidence that has been obtained since then. Perhaps the single most direct and informative study was the X-ray crystallographic analysis of the structure of the PRC. This was the first membrane protein to have its crystal structure determined to a resolution better than 3Å (Deisenhofer et al., 1985; Rees et al., 1989). The PRC is a complex of three integral membrane proteins L, M, and H, together with a *b*-type cytochrome peripheral protein. The L and M polypeptide chains are homologous to one another, each traversing the

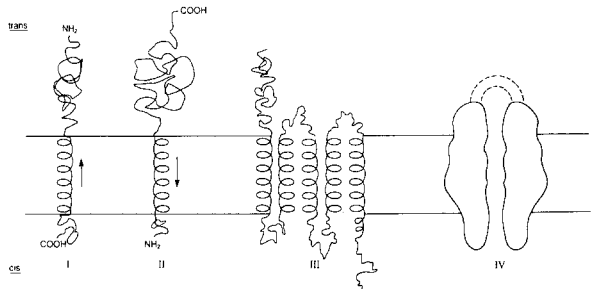

Fig. 2. The modes of membrane intercalation of the four main types (*I–IV*) of integral proteins so far elucidated. The external and cytoplasmic surfaces of the membrane are designated *trans* and *cis*, respectively. Type *III* proteins represent a collection of different topographies with different possible orientations of their N- and C-termini, and different numbers of transmembrane stretches, but without forming an aqueous channel across the membrane. Type *IV* proteins are channel-forming proteins, here shown in cross-section as having two homologous, but not necessarily identical, subunits defining the channel. The number of subunits, however, can be larger than two. The stippled arch indicates that the subunits may either be homologous domains of a single polypeptide chain, or independent polypeptide chains. (Reproduced, with permission, from the *Annual Review of Cell Biology*, Vol. 6, 1990.)

membrane five times; they can be classified as Type III proteins (Fig. 2). The H chain has a single transmembrane stretch, and is a Type I protein. With respect to the original predictions we made about membrane proteins that have so far been discussed, the relevant facts about PRC structure are: (1) of the nearly 150 ionic residues of the complex, not one is located in the membrane-interior regions of the complex; and (2) the membrane spanning domains are entirely in the α-helical conformation. These data provide the most definitive evidence supporting our proposals for the amphipathic structures of the molecules of membrane proteins, and for the helical conformation of their hydrophobic bilayer-embedded domains. Much other evidence is consistent with these ideas as well (Singer, 1990).

Bacterial Porins

The high resolution X-ray crystallographic structure of the porin from *Rhodobacter capsulatus* has recently been reported (Weiss et al., 1991). Porins are structurally unique integral proteins in the outer membranes of gram-negative bacteria that form aqueous channels through the membrane which are permeable to small molecular species. The remarkable feature of the structure of this protein (which exists as a homotrimer in the membrane) is that its transmembrane domain is entirely devoid of α-helix. Instead, it contains a 16-stranded antiparallel β barrel, in which each strand is connected to its two neighbors via interpeptide hydrogen bonds. The interior of the barrel forms the channel across the membrane, and the exterior surface of the barrel is partly in contact with the other monomers of the trimer and partly in contact with the lipid bilayer. Such a β barrel, as had been suggested earlier (Kennedy, 1978), is a structure that maximizes the numbers of interpeptide hydrogen bonds in the interior regions of the membrane, and therefore is a thermodynamically satisfactory alternative to a set of α-helices. Furthermore, whereas each membrane-embedded α-helical strand of most Type III and IV integral proteins corresponds to a stretch of ~20 consecutive non-ionic, mostly hydrophobic amino acids, each membrane-embedded strand of a β barrel can be of mixed amino acid composition.

Thermodynamics, Membrane Asymmetry, and Transmembrane Transport

Two factors, (1) the amphipathic structures proposed for membrane protein molecules with their hydrophilic domains protruding from the bilayer and (2) the highly unfavorable free energies required to move the ionic residues of the protein across the hydrophobic interior of the bilayer, suggested to us that the rotation of such proteins across membranes would be highly unlikely to occur at any significant rate. We realized that this restriction on the transbilayer rotation of membrane proteins had a direct bearing on the question of membrane asymmetry. Our laboratory (Nicolson & Singer, 1971; Hirano et al., 1972), along with many others (*cf.* Benedetti & Emmelot, 1967; Marchesi & Palade, 1967), had provided evidence that individual proteins of a membrane have a characteristic molecular orientation across the bilayer. It was proposed, therefore, that if after synthesis the molecules of membrane proteins were each initially inserted into the bilayer in a particular orientation, their inability thereafter to rotate across the membrane would ensure the structural asymmetry of the membrane. In this view, the asymmetrical orientation of membrane proteins is not the result of achieving an equilibrium distribution of the proteins across the membrane bilayer, but is on the contrary a nonequilibrium state. The focus of attention concerning membrane asymmetry was therefore directed to the mechanism of the proposed initial asymmetrical insertion of these proteins into the membrane, a subject that we take up later on.

The problem of the asymmetry of the phospholipids of membranes (Zwaal, Roelofsen & Colley,

1973) is more complex. The ionic and highly polar head-groups of a phospholipid molecule would be expected, for the reasons already discussed, to inhibit the direct transbilayer rotations of these molecules. Indeed, in pure lipid bilayers, phospholipid rotations are not detectable (Roseman, Litman & Thompson, 1975). However, in some cellular membranes under appropriate physiological conditions, transbilayer movement of phospholipids appears to occur. A particularly interesting situation arises during the biosynthesis of certain membranes, where the protein machinery for the synthesis of a particular phospholipid may be located exclusively on one side of a membrane, yet that phospholipid is present (not necessarily in equal amounts) in both halves of the bilayer. Rothman and Kennedy (1977) demonstrated in the case of bacterial membranes that transbilayer rotations of phosphatidylethanolamine (PE) did indeed occur from the cytoplasmic leaflet of the membrane (where the phospholipid was synthesized) to the exterior leaflet, but only during the time of exponential growth of the bacterial cells. In the stationary state, transbilayer movement of PE was not detected. One possibility to explain these results is that such transbilayer phospholipid movement is somehow mediated by particular proteins ("flippases") in the membrane, but no convincing evidence has as yet been obtained to identify such proteins. Furthermore, an explanation is required why such putative flippases in bacterial membranes function during exponential growth but not in the stationary state. Another possibility is that proteins are not directly involved in such phospholipid rotations, but that during exponential growth lysophosphatide intermediates of phospholipid synthesis, which are detergent-like molecules, achieve sufficiently large local concentrations so as to induce a local phase change (Cullis & de Kruijff, 1979) in the lipid within the bilayer. At such altered sites, phospholipid transfer across the bilayer could be expedited. Upon ceasing growth, the lysophosphatide concentration might decrease as phospholipid synthesis was completed; the local phase change would then be reversed, and transbilayer movement of the phospholipid would no longer occur. In this view, the distribution of phospholipids across a membrane might, at least transiently, be an equilibrium distribution, determined perhaps by the different charge distributions on the protein domains protruding from the two surfaces of the membrane (von Heijne, 1986).

We realized early on that the thermodynamic restriction on transbilayer rotations of membrane proteins was also significant for the mechanism of transport of ions, and small hydrophilic molecules like sugars and amino acids, across membranes. In the

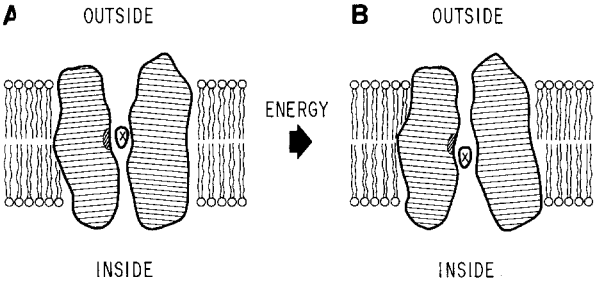

Fig. 3. A schematic representation of the mechanism for the transmembrane transport of an ion or a small hydrophilic molecule (×) via a specific Type IV integral protein (Fig. 2). In one state of the aggregate, the binding site for × (stippled) is accessible from one side of the membrane; in the other state the binding site faces the other side of the membrane. With an appropriate energy input, a quaternary rearrangement of the subunits, converting the first state into the second, powers the translocation of × across the membrane. (Reproduced, with permission, from the *Annual Review of Biochemistry*, Vol. 43, 1974.)

1960's, a popular model for such transport involved the "rotating carrier" mechanism. In this model specific membrane proteins bound a particular ion on one side of the membrane, then rotated across the membrane and released the ion on the other side. For the reasons discussed above, this rotation mechanism seemed entirely unlikely to us. Instead, we suggested (Singer, 1971) that transport might be mediated by proteins consisting of an aggregate of subunits that formed a water-filled channel down the central axis of the aggregate, with the channel spanning the membrane. [We have classified such pore-forming subunit aggregates as Type IV membrane proteins (Fig. 2)]. The surfaces of the subunits lining the channels could contain polar and ionic residues since they would be in contact with the water in the channel, but the surfaces in contact with the lipid interior of the bilayer would not contain any ionic residues. The subunit aggregate might exist in two main states (Fig. 3). In one state, an ion would become bound to a site within the channel facing one side of the membrane (Fig. 3*A*). In the other state (Fig. 3*B*), the conformation of the channel is altered; the ion-binding site now faces the other side of the membrane and suffers a decrease in ion-binding affinity, thus releasing the ion. Transition of the subunit aggregate from the former to the latter state, which corresponds to a quaternary structural rearrangement of the aggregate, would transport the ion across the membrane. Such a substantial quaternary rearrangement has the attractive feature that it might require only a small free energy input.

At the time that these proposals about membrane transport were made, no transport proteins had as

yet been isolated or characterized. An important transport protein that was under investigation by Nachmansohn and his colleagues during the 1960's was the acetylcholine receptor (AChR). AChR, present in the post-synaptic membrane at the neuromuscular junction, was thought to open a cation channel in response to binding acetylcholine, but in 1971 little was known about the structure of AChR or the mechanism of channel-opening. Our ideas about membrane transport led us to make the following specific proposal (Singer, 1971). After suggesting that AChR is a subunit aggregate as depicted schematically in Fig. 3, we proposed that "the binding of acetylcholine at a receptor site on one subunit 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." These predictions about the subunit structure and the acetylcholine-induced quaternary rearrangement of AChR have been borne out by subsequent experimental findings (*cf.* Unwin, Toyoshima & Kubalek, 1988).

A number of other transport proteins have been shown to be subunit aggregates (*cf.* Grenningloh et al., 1987; MacKinnon, 1991). In other instances, however, the transport protein is a single polypeptide chain, as in the case of the voltage-sensitive Na^+ and Ca^{2+} channels (Jan & Jan, 1989). With these proteins the amino acid sequence of the single chain appears to be divisible into four homologous, but not identical, domains. The clear implication is that each of the four domains is positioned within the membrane around a fourfold pseudosymmetry axis, with a transmembrane channel running down that axis. In other words, although such transport proteins are not aggregates of independent subunits, they are effectively similar to such aggregates structurally and probably functionally. (Such single chain Type IV molecules are schematically represented in Fig. 2 by the dash-lined bridge connecting the subunits.)

Our ideas about transport proteins also led us to propose (Singer, 1974) a structural model for the molecular mechanism of periplasmic transport systems in gram-negative bacteria (Ames, 1986); at the time, the mechanism was entirely unclear. This subject will not be dealt with in this article, but has been discussed in detail elsewhere (Singer, 1989). Suffice it to say, we predicted that certain integral membrane proteins would be found that would be essential to periplasmic transport which had not yet been discovered; these predictions and the structural model have turned out to be substantially in accord with later experimental evidence (Ames, 1986).

The Bilayer Couple Hypothesis

It occurred to us that the existence of membrane asymmetry has at least one other important consequence, namely that the two halves of a bilayer, while remaining coupled to one another, could respond differently to a perturbation. We called this the *bilayer couple hypothesis* (Sheetz & Singer, 1974), by rough analogy to the temperature response of a bimetallic couple. A particularly important perturbation is a shape change in a closed membrane. Geometrically, where a bilayer is curved, the areas of the two half leaflets of the bilayer must be different. Such bending of uniform bilayers requires energy (*cf.* Oster et al., 1989). Now, different phospholipid molecules can be assigned different molecular shapes (Cullis & de Kruijff, 1979); for example, glycolipids, because of their relatively large polar head groups, are roughly wedge-shaped molecules, whereas phospholipids like phosphatidylcholine, with smaller head groups, are more nearly cylindrical. The lipids could distribute in the plane of the membrane to minimize the bending energy in the bilayer, so wedge-shaped lipids might concentrate in the outer half leaflet of a convex-curved region of a bilayer, cone-shaped molecules in the inner half, and cylindrical molecules in adjacent planar regions. Differently shaped molecules of integral proteins may also distribute in the bilayer so as to minimize the bending energy.

These considerations apply to certain physiologically-relevant situations. For example, plasma membranes contain regions of high curvature where microvilli or dendritic extensions exist, or where buds or endocytic structures form. In addition, intracellular membranes such as those of different transfer vesicles, or the rim regions of Golgi saccules, show high membrane curvature. The lipids and integral proteins in the bilayer may therefore be distributed differently in curved and planar regions of membranes, just in order to minimize the bending energy. (Other factors, such as specific interactions of the membrane components with peripheral proteins, may also contribute to the redistributions of the components within the bilayer.) The formation of vesicles from planar membranes or the converse, the fusion of vesicles with planar membranes, are processes that involve net changes in membrane curvature, which may produce changes in the lipid composition of the membranes in accord with the bilayer couple hypothesis (Singer & Oster, 1992). In general, the biochemical consequences of membrane curvature have largely been overlooked by molecular cell biologists or have been discussed only in terms of peripheral protein assemblies, such as the clathrin complex (Pearse & Bretscher, 1981) attaching to the bilayer.

Another important application of the bilayer cou-

ple hypothesis was to the shape changes induced by the intercalation of amphipathic drugs and related molecules into asymmetric membranes (Sheetz & Singer, 1974). An interesting use of these ideas was recently made to the study of the molecular basis for the mechanosensitivity of certain ion channels in cells (Martinac, Adler & King, 1990).

Integral and Peripheral Proteins

In retrospect, the most daring proposal we made in the early 1970's has, paradoxically enough, since then become the blandest of conventional wisdom. This is the idea (Singer, 1971) that there are two very different kinds of membrane-associated proteins, integral and peripheral. Our notions about the amphipathic structures of membrane proteins led us to expect that such proteins would be insoluble in water because of their hydrophobic domains. On the other hand, investigators had found that certain membrane-associated proteins, once extracted from the membrane, were indistinguishable in properties from water-soluble serum or cytoplasmic proteins; examples are cytochrome *c* of mitochondrial membranes and spectrin of red blood cell membranes (Nicolson, Marchesi & Singer, 1971*b*). We therefore made the suggestion that whereas the molecules of most membrane proteins were amphipathic and embedded in the bilayer by their hydrophobic domains (the integral proteins), others were neither amphipathic nor embedded (the peripheral proteins), but were instead noncovalently attached to the hydrophilic domains of specific integral proteins where these protruded from the membrane into the aqueous phase. The proposals for the existence, the structural differences, and the modes of membrane association of integral and peripheral proteins were subsequently discussed in greater depth (Singer, 1974). In the ensuing years, these notions have become commonplace in membrane biology.

Some corollaries of these proposals were immediately realized. One such corollary was a molecular mechanism for collecting certain integral proteins together into domains within a membrane. If a self-aggregating peripheral protein were attached to a particular integral protein, as we proposed might be the case with the spectrin network underlying red blood cell membranes, it would have the effect of cross-linking and collecting the attached integral protein within the membrane. An interesting example of such membrane-domain formation involving the integral protein AChR was recently published (Phillips et al., 1991). Another corollary of the distinction between integral and peripheral proteins had to do with mitochondrial proteins. By 1970, the remarkable fact was known (*cf.* Ashwell & Work, 1970) that most of the proteins of mitochondria, including cytochrome *c*, were synthesized in the cytoplasm, whereas only a small fraction was synthesized inside the mitochondrion. Clearly the former proteins had to be imported across one or two membranes into the mitochondrion, but why were the latter proteins different? Recognizing that our proposed structure for integral proteins might make it difficult for them to pass through mitochondria without becoming embedded in the outer membrane by their hydrophobic domains, we suggested, or rather hinted (Singer & Nicolson, 1972), that those proteins that were synthesized inside mitochondria might be "only the integral proteins of the inner mitochondrial membrane." Although the facts discovered since then (*cf.* Hartl et al., 1989) make the situation more complicated than we projected in 1972, our proposal was clearly on the right track.

Membrane Structure and Membrane Fluidity

The picture of membrane structure that we developed in 1966 (Fig. 1*B*) was essentially two-dimensional, in cross-section. It did not directly address the third dimension in the plane of the membrane, which we recognized might involve two different kinds of structures. In one, the lipid would form the matrix of the membrane, with integral proteins more-or-less randomly dispersed within the lipid; in the other, protein-protein interactions would define the membrane matrix, with lipid present in the interstices between the proteins. We thought that the latter structure was likely to arise only when a single integral protein predominated in the membrane, as turned out to be the case in the membrane of the photosynthetic purple bacterium where bacteriorhodopsin was the predominant integral protein (Blaurock & Stoeckenius, 1971). Because most membranes appeared to be very heterogeneous in their protein composition, however, we favored the idea of a lipid matrix for the general case. One prediction that followed from such a mosaic structure of integral proteins embedded in a lipid matrix was that any particular protein should be randomly distributed in the plane of the membrane. Random distributions were then demonstrated by immunoelectron microscopy to be characteristic of two different proteins in the red blood cell membrane (Nicolson, Hyman & Singer, 1971*a*; Nicolson, Masouridas & Singer, 1971*c*). We also entertained the idea that the integral proteins might actually undergo diffusion in the plane of the membrane, and during discussions I raised during a visit to the Rockefeller Institute in February, 1971, Dr. Siamon Gordon informed me

about the remarkable paper by Frye and Edidin (1970) that had just appeared. These authors provided strong evidence that proteins could rapidly diffuse and intermix over long distances in the surface membranes of lymphocytes. The occurrence of such global diffusion of cell surface molecules was entirely explicable by our proposed mosaic structure of integral proteins embedded in a lipid matrix, if the lipid throughout the membrane existed in a fluid state (*cf.* Scandella, Devaux & McConnell, 1972). Combining this evidence together with our structural ideas, we proposed that most membranes were organized as fluid mosaic structures (Singer & Nicolson, 1972).

At about the same time, the global diffusion of proteins in membranes was demonstrated by an experimental technique different from that of Frye and Edidin, namely by the clustering, or "capping," of a membrane molecule that was induced by a specific bivalent antibody (Taylor et al., 1971). In retrospect, the fact that we did not encounter the phenomenon of global diffusion earlier in our own experiments has a curious twist to it. Our immunoelectron microscopic experiments (Nicolson et al., 1971*a*,*c*), alluded to earlier, were carried out with red blood cells rather than lymphocytes. This turned out to have kept us from observing the capping phenomenon, because the membranes of mammalian red blood cells are unique among cell membranes by virtue of the restrictions that are placed on the global diffusion of its surface molecules by the spectrin complex underlying the membrane. In our antibody labeling experiments, we therefore observed that antigens were dispersed in the plane of the membrane, instead of being collected into a cap.

Membrane Fluidity and Membrane Functions

As a corollary of the fluid mosaic structure of membranes, we recognized that the lateral mobility of components in the fluid membrane brought a new dimension to membrane biology and biochemistry, and was likely to have many novel functional consequences. In its most general form, the central idea involved was stated as follows (Singer & Nicolson, 1972): "The physical or chemical perturbation of a membrane may affect or alter a particular membrane component or set of components; a redistribution of membrane components can then occur by translational diffusion through the viscous two-dimensional solution, thereby allowing new thermodynamic interactions among the altered components to take effect." Many instances can now be cited that confirm and elaborate upon this general suggestion. One of the most interesting involves the activation of cells by polypeptide hormones or growth factors. The specific receptors for different growth factors are generally Type I integral proteins (Fig. 2), whose external hydrophilic domain contains the binding site for the growth factor, and whose cytoplasmic hydrophilic domain contains an enzyme active site (often for a protein tyrosine kinase). The binding of the growth factor on one side of the membrane activates the latent enzyme activity on the other side. However, as was noted earlier, with Type I integral proteins, the external and cytoplasmic domains are most likely conformationally independent of one another, so the mechanism of this ligand-induced activation has been obscure. In recent years, however, evidence has been provided that: (1) the binding of the growth factor to its receptor induces a homo-dimerization of the originally monomeric receptor molecules by diffusion within the membrane; and (2) this dimerization is critical to the process of enzyme activation in the cytoplasmic domain of the receptor (Schlessinger, 1988; de Vos, Ultsch & Kossiakoff, 1992).

This dimerization mechanism for signaling events mediated by Type I integral proteins is different from that mediated by Type III proteins (Fig. 2). With Type III molecules, as discussed earlier, the exterior, membrane-interior, and cytoplasmic domains are all conformationally interdependent, in contrast to the case with Type I molecules. The binding of a ligand to the exterior domain of a Type III molecule can cause a conformational change that affects the structure of the entire molecule, including the cytoplasmic domain. The β-adrenergic receptor, for example, upon binding epinephrine at its external domain undergoes a conformational change that confers upon its cytoplasmic domain a binding affinity for a G-protein (O'Dowd, Lefkowitz & Caron, 1989).

Another important process for which we suggested that membrane fluidity is critical is cell-cell adhesion (Singer, 1976). We proposed that under appropriate circumstances, the interaction of cell surface ligand molecules on one cell with their specific cell surface receptors on a second cell, can result in a mutual clustering of the two kinds of molecules into the membrane regions at the cell-cell contact. This clustered intercellular bonding is the basis for the strong intercellular adhesion (Singer, 1992*b*).

The Biogenesis of Membranes

Before 1970, there was little understanding about how the components of membranes might be assembled. The fluid mosaic model raised, however, some

searching questions about membrane biogenesis (Singer, 1971). First of all, it was appreciated that a mosaic structure would require only a loose coupling between the synthesis of the lipids and integral proteins of a membrane, and their subsequent incorporation into it. Pursuing the question of incorporation, we asked "how then do the integral proteins get into the membrane, particularly if, as nascent chains not yet bound to lipid, they would be very insoluble in the cytoplasm? . . . . How would a plasma membrane protein get incorporated exclusively into the plasma membrane and not into the outer membrane of a mitochondrion if diffusion through the cytoplasm were involved?" We then speculated that "a ribosome, or polyribosome, 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 first being solubilized and not necessarily synchronously with lipid."

In retrospect, these thoughts and speculations were clearly on the right track, but could not be taken much further considering the knowledge available at the time. The discovery of signal sequences a few years later (Milstein et al., 1972; Devillers-Thiery et al., 1975) supplied the critical clue to the puzzle. It is the signal sequence that provides the specificity of membrane attachment, and that initiates the process of polypeptide translocation across (Blobel & Dobberstein, 1975), or intercalation into (Rothman & Lenard, 1977), the membrane. Neither translocation nor intercalation of a long polypeptide chain is likely to occur directly through the lipid of a membrane because of the severe thermodynamic restraints placed upon transferring ionic amino acid residues through the hydrophobic interior of the bilayer. A protein machinery is required. The first molecular model of this protein machinery (Blobel & Dobberstein, 1975; Blobel, 1980) was based on our proposal that there exist integral proteins made up of subunit aggregates which form a transmembrane channel down the central axis of the aggregate (Type IV proteins, Fig. 2). At the present time, much progress is being made in the identification of the components of the protein machinery mediating the translocation across different types of membranes but no consensus has yet arisen about the detailed structure or the functional mechanism of the machinery. Our recent mechanistic proposals concerning this machinery have been discussed in a review (Singer, 1990). We support the suggestion (Blobel, 1980) that the insertion of the polypeptide chains of integral proteins into a membrane occurs by a stepwise sequential process which is initiated at the N-terminal signal sequence of the chain, and we suggest how this could be achieved using a Type IV protein translocation machinery (Singer, Maher & Yaffe, 1987*a*,*b*). Such sequential insertion accounts for the 100% α-helical character of the bilayer-embedded hydrophobic domains of most integral proteins, because as each transmembrane domain is inserted it adopts the α-helical conformation. Insertion via the protein translocation machinery also ensures that the protein molecule becomes integrated in a characteristic orientation in the membrane. As we discussed earlier, such oriented insertion is most likely the basis for the asymmetry of membranes.

Epilogue

The last few decades have seen tremendous advances in our understanding of membrane structure and function, and we are gratified to have taken part in this progress. When we entered the field, membranes were esoteric cellular entities on the outskirts of biochemistry. No membrane proteins had as yet been isolated and characterized, and the understanding of membrane structure was primitive. In the 1960's, those faculty members in the chemistry departments of this country's universities who worked on membranes could be counted on the fingers of one hand. Today, there is hardly a major chemistry department without at least one such faculty member. Despite this explosion of interest and knowledge, however, much remains to be done. One major problem is that of the structures of integral proteins. One can get the impression that we now know a great deal about the three-dimensional structures of these proteins. In fact, however, this is only true for a very few integral proteins whose structures have been analyzed by X-ray crystallography to a resolution of 3 Å or better. This has required the preparation of satisfactory three-dimensional crystals of pure membrane proteins. Electron crystallography of two-dimensional crystals has made enormous strides, and the structures of bacteriorhodopsin (Henderson et al., 1990) and the light harvesting complex of plants (Kühlbrandt & Wang, 1991) have been determined to 6 Å resolution or better. But such resolution is still not good enough to establish the locations of individual amino acid residues in the structure. The problem of structure determination is inherently simpler for Types I or II integral proteins, whose hydrophilic domains can, at least in principle, be isolated and crystallized from aqueous solutions and studied in conventional fashion. These hydrophilic domains can be obtained either by pro-

teolytic cleavage from their hydrophobic membrane-embedded domains (*cf.* de Vos et al., 1992) or by means involving genetic engineering. However, for Types III and IV proteins, the entire molecule must be subjected to X-ray crystallographic analysis, which usually requires that the pure protein be solubilized in a suitable non-ionic detergent complex, and then that this protein-detergent complex be crystallized from an aqueous medium. Only one Type III protein complex [the bacterial PRC mentioned earlier (Deisenhofer et al., 1985; Rees et al., 1989)] and one Type IV species [bacterial porin (Weiss et al., 1991)] have so far been successfully analyzed at high resolution. This has not been for lack of trying (*cf.* Kühlbrandt, 1988; Michel, 1991). An outsider to this field senses that the problem of making suitable crystals containing Types III or IV proteins is intrinsically more difficult than is the case with water-soluble proteins. This difficulty deserves more intensive analysis and study, given the importance of the integral protein structure problem to the future of membrane biology. It may require that general methods be developed whereby membrane proteins are appropriately modified by site-directed mutagenesis and recombinant DNA methods in ways that permit a more successful crystallization of these proteins without significantly altering their structures. The determination of the detailed structures of a wide variety of Types III and IV integral proteins will open up a vast area of membrane biology that is not now accessible to definitive study. This memoir has mainly focused on the membrane biology of the past, but the future holds promise of enormous adventure.

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