The lipid bilayer membrane and its protein constituents

Janice L. Robertson

In 1918, the year the *Journal of General Physiology* was founded, there was little understanding of the structure of the cell membrane. It was evident that cells had invisible barriers separating the cytoplasm from the external solution. However, it would take decades before lipid bilayers were identified as the essential constituent of membranes. It would take even longer before it was accepted that there existed hydrophobic proteins that were embedded within the membrane and that these proteins were responsible for selective permeability in cells. With a combination of intuitive experiments and quantitative thinking, the last century of cell membrane research has led us to a molecular understanding of the structure of the membrane, as well as many of the proteins embedded within. Now, research is turning toward a physical understanding of the reactions of membrane proteins and lipids in this unique and incredibly complex solvent environment.

Introduction

Organisms live and thrive in a wide range of chemical environments with varying physical conditions. Life has been found at freezing temperatures, thermal vents, extreme acidic and alkaline conditions, and a wide range of salt concentrations. This diversity is possible because the cellular membrane presents a robust barrier to the external world, setting the stage for exquisite control of the cell's internal solutions. First and foremost, the membrane must be a barrier to salts, protons, and charged molecules to maintain proper biochemistry. In addition, if the membrane is capable of separating charge, like a low dielectric insulator, then it can also store electrical as well as chemical potential energy to fuel future biological work. Finally, the membrane must be capable of changing its shape to accommodate the increasingly complex requirements of biology, and therefore, it must be fluid in nature. It is remarkable that all of the physical properties listed above are satisfied by a two-molecule-thick layer of self-assembled oil and even more amazing how proteins have evolved to function in this dramatically different physical environment. The lipid bilayer provides a robust solution for a physiological barrier, and its simplicity is a foundation for the origins of life and evolutionary diversity (Schrum et al., 2010).

In this Milestones article, I review some of the key experiments that led to our current understanding of the cell membrane as a lipid bilayer that solvates proteins that span the membrane. It is a daunting task to go over this enormous body of work, and so this article presents a mere sampling of the research. For those desiring a deeper review of the history, I refer you to several other resources (Tanford, 2004; Lombard, 2014; Stillwell, 2016). In this paper, I first discuss how our understanding of cell membranes went from invisible boundaries to fluid lipid bilayers. Next, I highlight the path to identifying a new class of membrane proteins that reside and function within hydrophobic lipid bilayers. Finally, I discuss current research of membrane proteins in lipid bilayers, highlighting the key challenges and complexities of studying proteins in a solvent environment that is self-assembling and has its own structural properties and increased chemical complexity. The past 100 years take us from a time when we did not know the lipid bilayer and integral membrane proteins existed to the present, where the structure of membrane proteins, even in the context of the cell membrane, is becoming more and more accessible. Throughout this time, the *Journal of General Physiology* has been a pivotal resource for the presentation and discussion of research that is the foundation of the field of cell membrane biophysics.

Department of Molecular Physiology and Biophysics, The University of Iowa, Iowa City, IA.

Correspondence to Janice L. Robertson: janice.robertson@wustl.edu;   J.L. Robertson's present address is the Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis, MO.
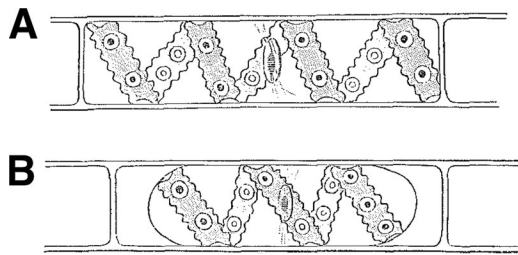
Figure 1.   **Plasmolysis reveals invisible barriers within living cells. (A and B)** A normal *Spirogyra* cell (A) and the cell during plasmolysis (B). From Overton (1895), Fig. 1 is adapted from *Vierteljahresschr. Naturforsch. Ges. Zürich.*

Interrogating invisible boundaries
Membrane? What membrane?

Equipped with a light microscope, Robert Hooke examined the intricate assemblies of numerous biological samples, documenting these close-up observations in his book, *Micrographia* (Hooke, 1665). One of the samples he examined was a material that was extracted from the underlayer of bark from a cork tree. He observed that the tissue was composed of a network of pores with thin "Interstitia, or walls" reminiscent of the "thin films of Wax in a Honey-comb." He referred to these pores as "cells" and proposed that other tissues, in both plants and animals, may be organized in a similar fashion. He extensively studied animal structures such as the valves of the heart and veins but did not see any similar cellular compartmentalization. We now know that the reason he could visualize the cellular structures of cork but not animal cells was because he was seeing the dried up cell walls, easily visible by eye using a microscope. But even still, in plant cells, it was apparent that there was another invisible bounding mechanism that encapsulated the cytosolic solution of protein, salts, and small molecules. This was observed in some cells, where the protoplasm appeared to pull away from the cell wall and also contain the vacuoles and organelles within (Fig. 1). What explained this encapsulation? The prevailing theory for hundreds of years was that the protoplasm was dense and colloidal, rich in protein and other biological molecules, which made it behave as a gel (Lombard, 2014). It was proposed that when this gel came into contact with the extracellular solution, there was

a hardening at the contact layer, perhaps through aggregation of the colloidal matrix. For a modern-day example of this, consider the popular molecular gastronomy technique known as spherification (Fu et al., 2014). This is a method that is being used in the food industry to produce artificial caviar or the juicy popping bubbles found in bubble tea. These spheres are constructed by dropping solutions containing a sodium salt of the carbohydrate polymer alginate into calcium chloride. At the interface, calcium binds to the alginate and stabilizes the polymer network to form a hardened shell enclosing the liquid of interest (Fig. 2 A). Therefore, there is a chemical precedent for this, but the question remained whether this was the actual mechanism of encapsulation in biological cells.

Plasmolysis and semipermeable membranes

In the second half of the 19th century, a simple experimental idea would introduce a new hypothesis about the bounding mechanism of cells (Tanford, 2004; Lombard, 2014; Stillwell, 2016). In 1855, Carl von Nägeli and Karl Cramer reported their observations that plant cells, when placed in hypertonic solution, set by increasing salt or sugar concentrations, would result in the protoplasm shrinking away from the cell wall (von Nägeli and Cramer, 1855). Conversely, when the cell was placed in a hypotonic solution, the cytosol would expand to the point of bursting. They referred to these experiments as plasmolysis studies (Fig. 1) and interpreted the results as a demonstration of a pliable barrier, capable of adapting its shape to the surrounding vessel: in this case, the cell wall. Finally, they demonstrated that vacuoles, when isolated from the cell, followed the same behavior, and the volume changes here were reversible. Wilhelm Pfeffer continued these studies and proposed that a skin, or plasma membrane, existed that covered the exposed surface of the protoplasm (Pfeffer, 1877). At that time, he suggested this membrane was similar to artificial copper ferrocyanide membranes that had been recently discovered by Moritz Traube (Traube, 1867). These were simple studies, ones that any young student can carry out today, yet the implications of the findings were profound. The results provided evidence that the barrier acted like an invisible skin, selectively allowing for the passage of water in and out of the cell, although limiting the permeability of salts and sugars.
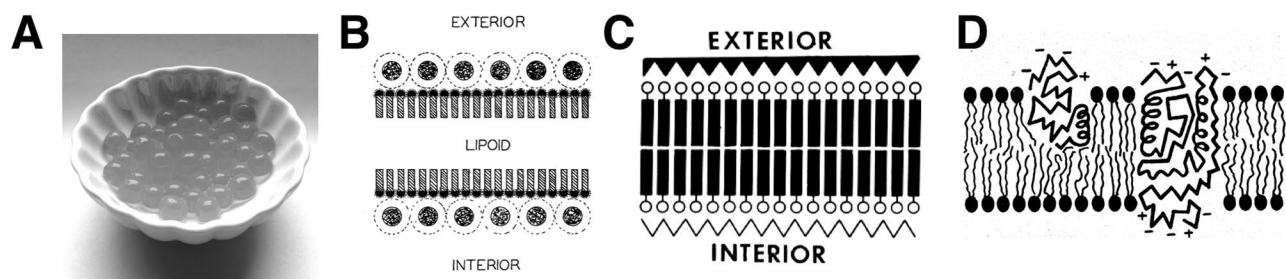

Figure 2.   **A brief history of cellular barriers. (A)** The protoplasmic colloid model. The barrier is a hardened shell that forms when the dense colloidal protoplasm makes contact with the extracellular solution. Pictured here is artificial caviar made by the analogous process of spherification. Photo courtesy of J.L. Robertson. **(B)** The paucimolecular model of Davson and Danielli, where the cell barrier is modeled as a lipid bilayer with a lipoid core flanked by layers of polar and charged proteins. From Danielli and Davson (1935), Fig. 2 B is adapted with permission from the *Journal of Cellular Physiology*. **(C)** The unit membrane model of Robertson, indicating the train track–appearing lipid bilayer that forms a continuous membrane around the cell. From Robertson (1981), Fig. 2 C is adapted with permission from the *Journal of Cell Biology*. **(D)** The fluid mosaic model of Singer and Nicolson, showing a lipid bilayer with integral membrane proteins responsible for cellular permeability. From Singer and Nicolson (1972), Fig. 2 D is adapted with permission from *Science*.

A lipid impregnated membrane

The early days of plasmolysis research were limited to qualitative visualization of changes under a microscope. Some studies aimed to quantify the changes by isolating the cells and weighing them before and after the osmotic stress, which sounds like a challenging task. However, Charles E. Overton would contribute significantly to our understanding of the membrane barrier by applying the osmometric technique with the understanding that the total osmotic pressure is equal to the sum of partial pressures in solution (Kleinzeller, 1999). With this improved resolution in hand, he could apply a quantitative and extensive approach to plasmolysis studies. Over a short period of time, from 1890 to 1900, he studied nearly 500 different compounds for setting the osmotic gradient, including salts, sugars, and nonpolar molecules (Overton, 1895; Kleinzeller, 1997, 1999). One of the surprising findings was that aliphatic alcohols did not lead to changes in the protoplasm's volume. Using his large body of quantitative data, he built a physical model of the cell membrane to explain his observations, modeled after gas laws that were being developed at the same time. He proposed that the lack of the change in the cell volume was caused by the free equilibration of nonpolar or lipoid-like molecules across the cell membrane. With this, he postulated that the cell membrane itself must be composed of similar nonpolar molecules to support the free partitioning of these substances and at the same time provide a barrier to salts and sugars. Therefore, the cell membrane was lipid impregnated and a distinctly different chemical environment from that of the cell's protoplasm or the external solution in which the cell bathed. Most surprisingly, he even proposed that cholesterol and phospholipids could be candidates for the nonpolar chemicals composing the membrane. Finally, he investigated a series of nonpolar molecules that were well known for their activity in humans: general anesthetics. In parallel research with Hans Horst Meyer, they both found that the propensity for an anesthetic to partition into olive oil strongly correlated with its efficacy in the clinical setting (Meyer, 1899; Overton, 1901), supporting the idea of a lipid-filled nonpolar barrier to cells (Fig. 3). The work of Overton and Meyer is a fine example of biophysics-based translational research at the turn of the 19th century. Although debates about the actual composition of the cell membrane persisted over the years, Overton's findings would end up standing the test of time. In 1976, Alan Finkelstein clarified these findings by carrying out a series of experiments studying the partitioning and diffusion of different solutes into nonpolar solvents. By studying solutes of different structures and sizes, he demonstrated that membrane permeability follows a coupled solubility–diffusion mechanism (Finkelstein, 1976).

Surface physics meets cell biology

At the same time as the plasmolysis studies, the study of surface physics was developing (Roberts, 2013). Benjamin Franklin, in the late 1700s, was one of the first to describe the behavior of a drop of oil to spread out thinly on a body of water (Tanford, 2004). In 1890, Lord Rayleigh reported his investigations on contamination of water surfaces by olive oil and estimated that the thin film was formed by a lipid layer of 10–20 Å in thickness (Rayleigh, 1889). This work sparked the interest of Agnes Pock-
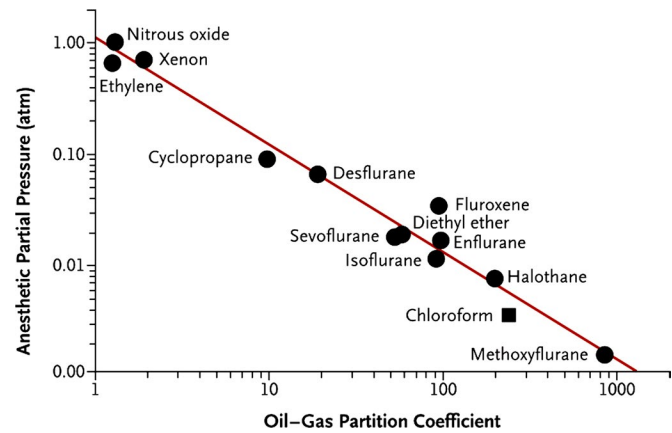

Figure 3. **The Meyer–Overton correlation.** Anesthetic efficacy in a clinical setting correlates strongly with the partitioning ratio of a compound from the gas state into olive oil. Their findings suggested that cell membranes are also lipoid in nature. From Campagna et al. (2003), Fig. 3 is adapted with permission from the *New England Journal of Medicine*.

els, a young woman who spent her time at home, caring for her parents and the household. Although she did not formally attend school, her brother was a university student studying physics, and she was exposed to the literature and textbooks that he would bring home. In her daily kitchen work, she would observe the behavior of oil on water while she washed the pots and pans, and this made her wonder about the molecular structure and physical properties of these thin films. With clear passion and determination, she built an apparatus for measuring the surface tension of the thin oil films on water out of the kitchen pans that she was so familiar with. She contacted Lord Rayleigh, and in 1891, he helped her to publish her first independent paper and one of the first studies of surface tension (Pockels, 1892). Several decades later, Irving Langmuir adapted Pockels's apparatus for higher-resolution measurements to build the Langmuir trough and with this solidified the conclusion that olive oil forms lipid monolayers on the surface that are 13 Å thick (Langmuir, 1917).

Early ideas of a lipid bilayer

The development of the Langmuir trough provided the key equipment for high-resolution investigations of the structure of cell membranes. If membranes were formed by oil, as Overton's experiments indicated, then they should behave similarly to the olive oil experiments performed by Lord Rayleigh, Pockels, and Langmuir. In 1925, Evert Gorter and Françoise Grendel extracted cell membranes from erythrocytes to measure the surface properties of the cell membrane using the recently developed physics approaches. The choice of erythrocytes was key to this experiment because these cells do not contain major organelles, and so the lipid fraction extracted would be expected to represent only the plasma membrane. They used the Langmuir trough to measure the surface area of the lipid molecules composing the cell membrane (Gorter and Grendel, 1925) and discovered that the area was exactly half of that expected if the molecules formed a monolayer. This led them to conclude that the cell membrane was a lipid bilayer, formed by two layers of oil molecules. This was the first proposal of such a structure and somewhat serendipitous

considering their experimental approach was far from robust. Changes in the lipid extraction methods and surface pressure dependencies can alter the conclusions of the experiment (Bar et al., 1966), and so the debate between a monolayer, bilayer, or other structure remained for decades. Still, the seed of the idea of the cell membrane as a lipid bilayer structure was planted, which was a sufficient starting point.

In the same year, Hugo Fricke measured the electrical capacitance of intact erythrocytes in suspension (Fricke, 1925). With prior knowledge that the membrane was lipid impregnated, he used a reasonable assumption of the dielectric constant, $\varepsilon = 3$, and determined that the hydrophobic thickness of the cell membrane was 3.3 nm. In hindsight, this is a remarkably accurate measurement in agreement with higher-resolution methods used today like small-angle x-ray and neutron scattering (Kučerka et al., 2009). Perhaps more importantly, this experiment demonstrated that the cell membrane was an electrical barrier. Fricke was cautious not to overinterpret his results, but if we take the 10–20-Å monolayer thickness measured by Rayleigh, Pockels, and Langmuir, the data are in agreement with a lipid bilayer structure.

In 1935, James Frederic Danielli and Hugh Davson compiled the many results from Fricke, Gorter, and Grendel and others to develop the paucimolecular model (Danielli and Davson, 1935), i.e., a model with the minimum amount of necessary molecular detail. Technically, Fricke's results allowed for membrane structures from unimolecular to trimolecular thickness, while Gorter and Grendel's work suggested a bilayer with room for error. In the Danielli and Davson model, they propose a bilayer of ampipathic lipoid molecules filled with a nonpolar lipoid center (Fig. 2 B). On either side of the membrane is a protein layer at least one molecule thick adsorbed onto the lipoid surface because it was believed that proteins could not stably exist inside the membrane. In their model, it was these protein layers that were responsible for selective permeability, and they could form structures spanning the membrane to enable the passage of salts and sugars. This model consolidated many of the physical findings of the cell membrane, but the idea of the membrane structure as a lipid bilayer remained under heavy debate for many decades.

This changed in 1959, when J. David Robertson was studying the ultrastructure of myelin sheaths at the Nodes of Ranvier by electron microscopy. To improve resolution, he applied potassium permanganate ($KMnO_4$) to stain structures, which highlighted a common trilaminar unit—two dark lines separated by a light center—that was observed at the plasma membrane boundary and encapsulating every organelle (Robertson, 1959). He interpreted the dark lines as the adsorbed protein layers and the light center as the lipoid, similar to the model of Danielli and Davson. The observation of the same structure being visible for all cellular compartments led him to propose the unit membrane model (Fig. 2 C), where he postulated that all membranes possess the same molecular structure. At that time, he even asserted that all membranes within a cell were in contact with one another, forming one continuous barrier. Although this idea is incorrect, the main point is that the lipid bilayer structure is something that is ubiquitous within biology, and it is capable of combining and fusing under the right conditions. In 1971, several studies measured the structure of membranes by x-ray diffraction (Levine and Wilkins, 1971; Wilkins et al., 1971), with results consistent with the lipid bilayer backing up Robertson's imaging studies with high-resolution physical data. Finally, in 1972, Singer and Nicolson published their fluid mosaic model of the cell membrane (Fig. 2 D), which is essentially the model of the membrane that is used today (Singer and Nicolson, 1972). Although many students are introduced to this concept in a single figure in a biology textbook, it does not do justice to the comprehensive understanding of the membrane structure, membrane proteins, and the thermodynamics of the system as a whole that is presented in their paper.

The lipid bilayer is a completely conserved structure across all of biology. It provides a thermodynamically favorable solution to the physical problem of creating an electrical barrier. It is something that assembles spontaneously, driven by the hydrophobic effect, burying nonpolar acyl chains that are constrained by the polar and charged lipid head groups. The hydrophobic core provides an environment where water is rarely present, creating a low dielectric environment, where partial charges cannot rearrange to counteract the electrostatic field. Thus, the free energy required to transfer a charged particle from a high dielectric medium, like water, to the low dielectric of the membrane is highly unfavorable, making the leakage of ions prohibitive (Parsegian, 1969). Furthermore, this hydrophobic core is self-stabilized by interactions between the acyl chains of the phospholipid molecules. Thus, it is a barrier that possesses structural integrity but also the properties of elasticity and pliability that have been described. With the lipid bilayer, a cell builds a self-assembling, self-repairing, practically impermeable barrier for charged particles and ions, enabling the storage of information and potential energy to fuel biology's work. It provides a natural and comprehensive solution, basically for free, and lets biology focus on the harder problem of enabling selective permeability.

A new class of protein that resides in membranes
Membranes are both lipoid and proteoid in nature
Plasmolysis studies also paved the way toward understanding the species responsible for selective permeability. Two of the pioneers in this field of work were Jacques Loeb and W.J.V. Osterhout, the first two editors of *JGP*. They independently performed some of the first studies of cell permeability in the presence of inorganic salts (Osterhout, 1911; Loeb, 1912). In the first of these studies, Osterhout repeated Overton's experiments of plasmolysis in *Spirogyra* but this time studied the cells over a longer period of time. When placing the cells in a concentrated salt solution, he observed Overton's initial result that the protoplast shrank away from the cell wall. However, over a longer time period, he observed the protoplast returned to its original shape and volume, which meant that the membrane was actually permeable to these inorganic ions. He went on to conclude that this must mean that the membrane was not composed of lipoid as previously asserted but instead was proteoid in nature. Perhaps this was too strong a conclusion given prior results, yet it highlights several truths about permeability across cell membranes. First, the passage of charges across the membrane must be electrostatically supported by protein, and second, membranes actually contain a large fraction of protein. A comprehensive review of permeability, and
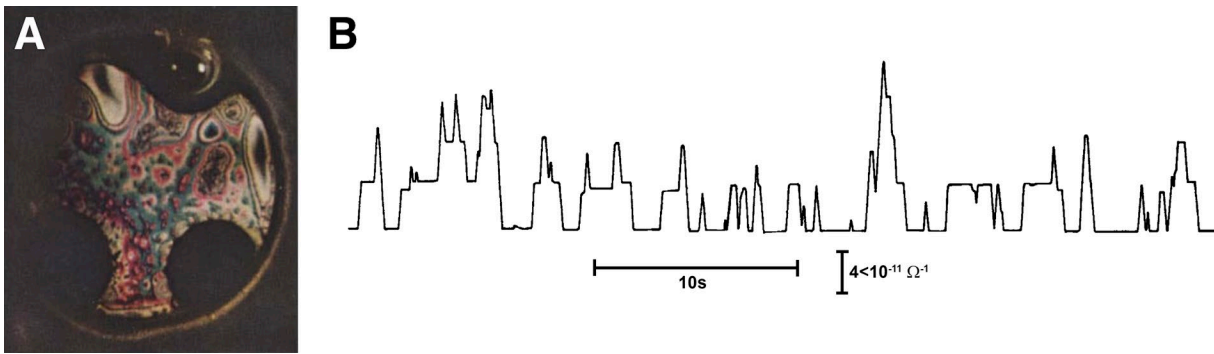
Figure 4. **Electrical reconstitution of membrane permeability. (A)** The BLM preparation for electrical measurements of permeability, adapted from Tien (1968). **(B)** The first single-channel recording of Gramicidin in lipid bilayers. From Hladky and Haydon (1970), Fig. 4 is adapted with permission from *Nature*.

more on the work of Loeb and Osterhout, can be found in a recent Milestone article in *JGP* (Hille, 2018). In the following sections, I highlight a few key studies that led to the solidification of integral membrane proteins as a new class of hydrophobic proteins that are responsible for the proteoid nature of cell membranes.

Ubiquitous Na⁺ and K⁺ gradients

The earliest ideas of proteins existing inside the cell membrane came from deductions based on physiological observations. In many cell types studied, Na^+ was observed to be at a higher concentration outside of the cell, while K^+ was found to be higher inside. In 1941, R.B. Dean proposed that to maintain these physiological concentrations, there must be a pump responsible for actively moving these ions against their gradients and that it may reside within the cell membrane (Dean, 1941). In 1957, Peter Mitchell supported this idea, proposing the existence of translocases, enzymes that catalyze substrate transport across the membrane (Mitchell, 1957), and introduced models of integral translocases with alternating access transport mechanisms. The discovery of the Na^+/K^+ ATPase by Jens Skou in 1957 (Skou, 1998) identified a specific membrane protein responsible for active transport; however, it still remained unclear as to how this protein was associated with the cell membrane. At that time, both the Davson–Danielli and Robertson membrane models did not account for protein inside the lipoid core as polar and charged protein molecules were expected to destabilize the membrane structure. Further evidence would be required before it would become accepted that proteins could span the hydrophobic membrane.

Isolated bilayers and black lipid membranes (BLMs)

A key development along the path to identifying integral membrane proteins was the discovery that one could purify fragments of membrane and reconstitute their electrical permeability behavior. In 1962, the first BLM (Fig. 4 A) experiments were conducted, in which a membrane was reconstituted outside of any cellular structure while maintaining similar electrical properties to intact cell preparations (Mueller et al., 1962). Higher-resolution BLM experiments would later reveal discrete, step-like addition of currents that contribute to the overall electrical permeability (Bean et al., 1969) indicating that whatever was responsible for the permeability, the behavior could be resolved in a quantal

manner. Finally, in 1970, the first single-channel recording was measured by adding the antibiotic peptide Gramicidin to a lipid bilayer (Hladky and Haydon, 1970). Gramicidin showed conductance behavior that was step-like, with constant step magnitudes (Fig. 4 B). With this, permeability could be attributed to the peptide itself, and the macroscopic behavior dissected into a sum of individual channel conductances in the membrane. This led to the idea of proteins in the membranes facilitating membrane transport by creating "permanent or transient modifications of the bilayer structure."

However, this was not enough to deduce that the protein was spanning the membrane. For example, Gramicidin could be acting as a rapid carrier, shuttling ions across the lipid bilayer, as is the case for ionophores like valinomycin. Alternatively, the protein could be modifying the membrane in some defined way that facilitates the passage of ions, akin to the ideas presented in the past, e.g., the Davson–Danielli model. To get to the idea of actual integral membrane proteins with defined structures, parallel efforts in membrane protein biochemistry were pivotal. It was long known that biological cell membranes were composed of proteins in high amounts; however, purification of these proteins led to an interesting result. Proteins obtained from the membrane fractions were mainly insoluble in water and often aggregated (Richardson et al., 1963). This suggested that the proteins that associated with membranes were somehow different in their physical nature compared with the vast number of soluble proteins studied so far. The next big piece of evidence came from electron microscopy freeze-fracture studies that split the lipid bilayer between the two leaflets. This directly showed that there were defects in the membrane that would appear as a positive signal in one leaflet, while leaving a negative impression, or hole, in the opposite layer (Moor and Mühlethaler, 1963). This provided the necessary evidence that proteins associated with membranes were in fact embedded in the lipid bilayer. With all of this accumulating evidence, Singer and Nicolson proposed the mosaic part of their fluid mosaic model (Singer and Nicolson, 1972), the idea of integral membrane proteins that are hydrophobic in nature and reside stably within the hydrophobic lipid bilayer. With further research in purification of membrane fractions and functional reconstitution (Miller and Racker, 1979), the idea of integral membrane proteins acting as ion channels, transporters, active pumps, and receptors soon became accepted.

Seeing is believing

As was the case with Robertson's electron microscopy studies, visual evidence is often required to remove remaining doubts. Fortunately, for the study of integral membrane proteins, there was a serendipitous source of highly ordered proteins in membranes that could be reasonably obtained from a type of archaea, *Halobacterium halobium*. These halobacteria were discovered to contain large, visible patches of purple membranes (Stoeckenius and Rowen, 1967). Dieter Osterhelt and Walter Stoeckenius developed methods for isolating these membrane fragments that contained nearly pure amounts of the protein bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971). These preparations resulted in protein that was already in ordered two-dimensional crystals within the membrane, and with this, in 1975, Richard Henderson and Nigel Unwin reported the first structure of an integral membrane protein of bacteriorhodopsin at 7 Å (Henderson and Unwin, 1975). It would take a decade before an atomic resolution structure of another membrane protein would be determined, with the delay being attributed to a fundamental biochemical challenge of membrane proteins. Protein crystallography requires the formation of highly ordered three-dimensional crystals from protein in solution. How, then, can membrane proteins be made soluble when they are hydrophobic and prone to aggregation? The solution came via the use of detergents and micelles as membrane mimetics (Helenius and Simons, 1975; Tanford and Reynolds, 1976). Detergent has the capability of extracting protein from the membrane, replacing the surrounding lipid, and, in some cases, supporting the membrane protein in a folded and functional state. With this key development, in 1985, Johann Deisenhofer, Harmut Michel, and others reported the first atomic-resolution, 3.0-Å crystal structure of the photosynthetic reaction center of *Rhodopseudomonas viridis* (Deisenhofer et al., 1985). With the ability to handle membrane proteins in solution, biochemical optimizations became tractable, and along with that came more structural information. In 1991, the structure of the bacterial outer membrane porin OmpF was solved at a remarkable 1.8-Å resolution (Weiss et al., 1991), on par with the resolution being obtained for soluble protein structures. In 1998, Rod MacKinnon and his laboratory determined the first structure of a potassium ion channel, revealing the elegant pore structure conferring selective K^+ permeability (Doyle et al., 1998). Since then, many structures of channels, transporters, pumps, and receptors have been solved, and today, they arrive at a steady pace, especially with recent technological developments in cryo-electron microscopy and single-particle reconstruction (Hendrickson, 2016). Although resolution is often lower for membrane proteins compared with soluble proteins, it is not an absolute fact, because many structures of membrane proteins have improved in resolution over time. One remarkable example is the structure of the yeast Aquaporin Aqy1 solved to a sub-angstrom 0.88-Å resolution (Eriksson et al., 2013). Still, it remains true that our knowledge of the structures of membrane proteins, and particularly eukaryotic membrane proteins, falls well behind that of soluble proteins and that this is a consequence of the inherent biochemical challenges associated with expressing and purifying stable membrane proteins.

The physical behavior of membrane proteins in membranes

As stated by Singer and Nicolson, the membrane is a "two-dimensional liquid-like solution of monomeric or aggregated integral proteins … dissolved in the lipid bilayer" (Singer and Nicolson, 1972). Yet, the membrane solvent is much more complicated than the other biological solvent that we are used to thinking about, i.e., water. The lipid bilayer has a defined structure and along with it has macroscopic material properties. The chemical composition of membranes is also extremely diverse. In the past this has been interpreted as lipids playing a passive role in membrane protein reactions, merely providing the sea of solvent in which the protein floats about. Following this, many studies of membrane protein biochemistry do not involve lipid bilayers but instead study the protein in detergent micelles, a practical necessity for purification in the aqueous state. This approach is well justified if the protein's function is maintained in a micellar environment, which can be the case when it is dominated by the intrinsic protein structure. However, it cannot be denied that standard methods of membrane protein purification strip away the natural solvent, placing the protein into an environment that could miss essential chemical or physical properties of the original lipid bilayer. Therefore, the reactions of membrane proteins, whether it is ligand binding, conformational stability, folding, or oligomerization, all depend on the free energy of the system as a whole, and this means in the context of the lipid bilayer.

Function and structure depends on specific lipids

Although the lipid bilayer structure is generally constrained to the 3-nm hydrophobic core measured by Fricke, it exists across biology with practically infinite chemical compositions that vary molecular head group and acyl chain chemistry. This diversity appears to be a result of an organism's need to maintain an electrical and physical barrier while preserving membrane fluidity under changing environmental conditions (Siliakus et al., 2017). Along with this diversity comes an apparent ability for specific lipids to regulate membrane protein function. Early studies of membrane protein reconstitution found that not all lipid bilayers were equivalent in terms of protein function and stability (Miller and Racker, 1979). As a result, many studies emphasized the use of lipid compositions as close to native conditions as possible. Furthermore, specific lipids were identified as essential for the proper gating activity, most notably the phosphoinositides (PIP_2; more on this particular lipid as a signaling molecule is described in the recent *JGP* Milestone review [Hilgemann et al., 2018]). Simply put, function, or lack thereof, is the strongest piece of evidence that particularities in lipid chemical species are important for the biological role of these proteins.

From a structural perspective, many high-resolution crystal structures of membrane proteins have resolved specific lipids directly associated with the protein. In some of these, the lipids were added during purification, and they became ordered during crystallization. However, in other examples, the lipids appear to have come along for the ride, remaining bound even in the harsh conditions of detergent extraction. One example is the K^+ channel KcsA, which was observed to bind a single negatively charged lipid per subunit (Fig. 5), and this lipid appears to be necessary for function (Valiyaveetil et al., 2002). High-reso-
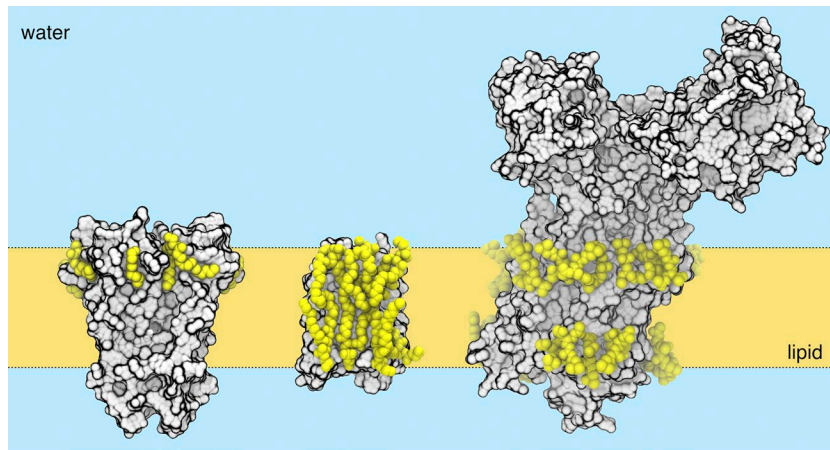
Figure 5. **Structural evidence of lipids interacting with membrane proteins.** From left to right, a POPG molecule bound to the K⁺ channel KcsA (Valiyaveetil et al., 2002), lipids bound to the interface of a single monomer of the water channel Aqp0 (Hite et al., 2010), and a ring of lipids resolved in the x-ray crystal structure of the Ca²⁺-ATPase by phase contrast imaging (Norimatsu et al., 2017).

lution electron crystallography structures of native crystals of Aquaporin-0 (Aqp0) show the proteins are organized in a lattice, stitched together by a layer of lipids (Fig. 5), with a high degree of specificity for certain lipids at different sites (Gonen et al., 2005; Hite et al., 2010). Recently, mass spectrometry analysis has provided more quantitative information about lipid binding and has been able to describe the specificity for interactions over other lipids (Laganowsky et al., 2014), as well as the ability for lipids to shift oligomeric distributions in detergent micelles (Gupta et al., 2017). Are these lipids that are found to interact with membrane proteins in structures or mass spectrometry analogous to crystallographic waters found in soluble proteins? Or do they represent something more like ligand binding in the context of the membrane? Electron spin resonance and fluorescence-quenching studies do not support the idea of long-lived lipids binding to membrane proteins in membranes (Lee, 2003). However, it is likely that further research is required with the new structural data in hand to provide context of the reactions in the membrane.

The lipid bilayer is a macroscopic material
In three dimensions, the lipid bilayer acts as an elastic material, with bulk properties of compressibility, elasticity, curvature, and lateral pressure profiles (Andersen and Koeppe, 2007). These properties are defined by the molecular interactions of the acyl chains and interactions with the head groups. At the same time, cellular membranes contain a high density of membrane proteins, and these act as inclusions or defects, shifting the energetics of the natural lipid bilayer. For instance, a membrane protein may be mainly hydrophobic but introduce a polar surface to the membrane's nonpolar core. Lipid molecules are flexible and compressible, and so they can rearrange to optimize the solvation structure around any protein embedded within the membrane and minimize the free energy of the system. As a result, membranes change their shape to minimize hydrophobic mismatch around proteins, but this is often associated with an energetic penalty. Hydrophobic matching of the membrane has been observed in molecular dynamics computer simulations, both coarse-grained models that allow for the substantial sampling and some all-atom models (de Jesus and Allen, 2013). Recently, experimental studies using x-ray solvent contrast modulation have resolved the structure of the lipid bilayer around

the Ca²⁺-ATPase pump in crystals (Norimatsu et al., 2017). They observe an accommodation of the lipids (Fig. 5), defined by interfacial Arg/Lys and Tyr/Trp residues, resulting in hydrophobic matching as has been predicted in fluorescence-quenching studies (Caffrey and Feigenson, 1981).

However, in many cases, it is not just the membrane structure that changes but the protein structure as well. The solution to hydrophobic mismatch comes via coupled structural changes of both the protein and the lipid bilayer, which together find the free energy minimum of the system as a whole. This relationship between membrane deformation and protein conformations has been described in the mattress model by Mouritsen and Bloom (1984). Here, the membrane is modeled as an elastic mattress made of springs, with its own equilibrium free energy (Fig. 6 A). A protein reaction, such as a conformational change or oligomerization, will depend on the intrinsic free energy of the protein as well as the external bias offered by the membrane. If a state of the protein sufficiently perturbs the membrane, then this could drive the conformational change or association of proteins (Fig. 6 B). Gramicidin provides an excellent example of how the membrane couples to the conformational stability of membrane proteins. Through a combination of fluorescence and conductance measurements (Veatch and Stryer, 1977) and NMR studies (Urry et al., 1983), it was determined that Gramicidin channel activity arises from the dimerization of two pores, one in each leaflet to form a continuous channel across the membrane (Fig. 6 A). However, the structure of the Gramicidin dimer is shorter than the full length of the membrane, and so dimer formation imposes hydrophobic mismatch on the surrounding lipid bilayer. With this, it was discovered that changing the lipid bilayer thickness, by changing acyl chain length, could shift the equilibrium of channel openings and closures (Andersen and Koeppe, 2007). Note that this sensitivity to hydrophobic mismatch is not true of all membrane proteins. In contrast, aggregation of bacteriorhodopsin was found to be generally insensitive to the lipid chain length except at bilayer thickness extremes (Lewis and Engelman, 1983). In other cases, other properties such as membrane strain may account for long-range conformational organization of membrane proteins (Fig. 6 C). Recently, it was demonstrated using coarse-grained molecular dynamics simulations that ATP synthase dimers organize into rows without direct
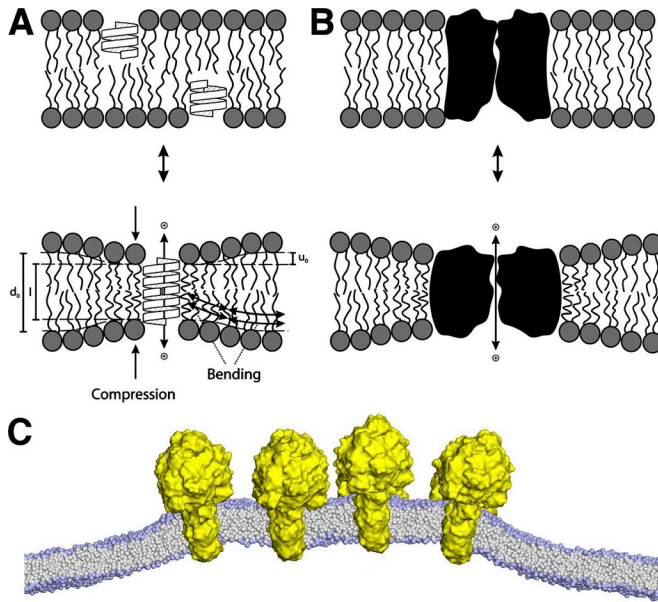
Figure 6. **Membrane protein stability depends on membrane energetics.** The lipid bilayer is a macroscopic material with elastic properties that affect membrane protein conformational stability and assembly. Membrane deformations have been shown to affect Gramicidin dimer equilibrium (A); ion channel gating (B), adapted from Lundbaek et al. (2004); and long-range assembly of ATP synthase dimers during the formation of cristae (C), from Anselmi et al. (2018), adapted with permission from *J. Gen. Physiol.*

protein contacts, but because of a minimization of membrane curvature deformations, and this may support the formation of cristae in mitochondria (Anselmi et al., 2018).

Self-assembly in the membrane

The reason why it took a long time to accept the idea of integral membrane proteins is that it was always a challenge to rationalize how polypeptide chains, which have polar backbones and charged side chains, could possibly be stable inside hydrophobic core of the lipid bilayer. We now know that this occurs because of the formation of secondary structural elements, α-helices and β-strands, that provide a hydrogen-bonding network that reduces the electrostatic penalty (White and Wimley, 1999). In addition, these proteins are lined with nonpolar, greasy residues that favor partitioning into membranes (Wimley et al., 1996; Hessa et al., 2005; Moon and Fleming, 2011). For α-helical membrane proteins, it has been proposed that assembly into lipid bilayers occurs following a two-stage model (Popot and Engelman, 1990). In stage 1, the polypeptide chain folds into a stable α-helix in the lipid bilayer, which can occur spontaneously for a small set of hydrophobic peptides (Wimley and White, 2000). In cells, this occurs during translation where the ribosome is targeted to the translocon channel, which facilitates the formation of α-helical structure and partitioning of nonpolar helices into the membrane (White and Wimley, 1999). In stage 2, the helices undergo equilibrium sampling of their different configurations to find the thermodynamically favorable and biologically relevant folded state (Fig. 7). In 1987, Jean-Luc Popot and Donald Engelman showed that proteolytic fragments of bacteriorhodopsin that were reconstituted into separate membranes could reassemble

into a folded, retinal-bound structure when the membranes were fused together. This provided strong evidence that the folded state of a membrane protein is a thermodynamically stable state in the membrane (Popot et al., 1987).

Beyond this, we have a limited understanding of what drives membrane proteins to assemble inside of the lipid bilayer. We know that membrane proteins are generally nonpolar to exist in the hydrophobic membrane. Yet, during assembly, we do not know why these greasy protein surfaces choose to form stable interactions with other greasy protein surfaces instead of the similarly greasy lipid solvent. To obtain a thermodynamic understanding, equilibrium studies of the second stage of membrane protein folding are required, but there are many technical challenges that arise. For soluble proteins, the study of reversible protein folding was made possible by using denaturants such as chemicals, temperature, or pH. The same approach is problematic for the study of membrane proteins in membranes. For example, a denaturing detergent like SDS would as much dissolve the membrane, thus changing the properties of the solvent environment. However, this is not impossible. Karen Fleming showed that using guanidine hydrochloride as a denaturant enabled the study of reversible folding of the OmpLA β-barrel protein from the aqueous state into POPC lipid bilayers for free energy measurements (Moon and Fleming, 2011). Refolding of α-helical proteins has been observed from denaturing solutions into lipid bilayers in a reversible manner (Riederer et al., 2018; Sanders et al., 2018) but only in the presence of detergents, thus complicating interpretation of the reference state.

Another approach to this problem is to study the reversible binding of protein segments, such as individual α-helices or protein subunits during oligomerization. This strategy simplifies the reaction so that equilibrium studies are tractable, and the reaction reports on the same physical driving forces. The major problem that arises when studying binding of membrane proteins in membranes is the limited protein signal. Because of this, studies are often restricted to high densities where hundreds of copies of protein are present within each liposome, which can saturate the reaction that is being studied. To get around this, strong affinity complexes have been studied at equilibrium with external biasing methods, such as redox driven disulfide exchange (Cristian et al., 2003) and steric trapping (Hong et al., 2010) to measure the influenza M2 tetramer and Glycophorin dimer, respectively. Alternatively, single-molecule microscopy methods can be used to study high-affinity binding reactions at dilute conditions. Using single-molecule photobleaching analysis, it was possible to measure the equilibrium dimerization reaction of the CLC Cl⁻/H⁺ antiporter by passive dilution in the membrane (Chadda et al., 2016, 2018). It should be noted that single-molecule methods are particularly well-suited for studying membrane protein assembly and dynamics. These experiments require minimal amounts of protein, and the studies are conducted away from high-density conditions where membrane proteins can be prone to nonspecific aggregation reactions. Single-molecule force microscopy approaches (atomic force microscopy and optical and magnetic tweezers) offer new ways of studying multihelix assembly of membrane proteins (Min et al., 2015, 2018; Yu et al., 2017). Although most of these studies are performed in detergent micelles
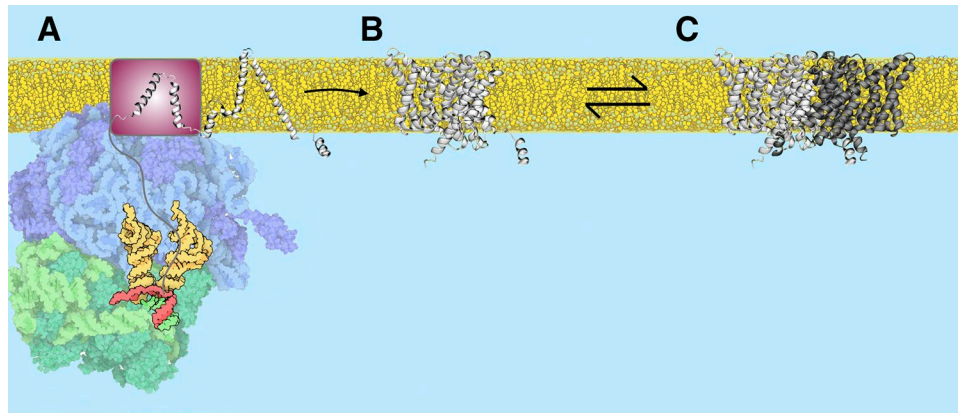
Figure 7. **Insertion and assembly of α-helical membrane proteins in membranes.** (A and B) Membrane protein folding follows the two-stage model (Popot and Engelman, 1990) involving synthesis and partitioning into the lipid bilayer, facilitated by insertases such as the translocon in cells (A), followed by the association of helices into the biological folded structure (B). Image of ribosome from Goodsell (2010), adapted from The Protein Data Bank. (C) Association of subunits or single helices provides a simplified model for studying the thermodynamics of membrane protein association in membranes.

or bicelles, it appears to be only a matter of time before these proteins can be studied in lipid bilayers.

The inherent complexity of the membrane

In cytosolic and extracellular solutions, there is only one solvent that is relevant to biological reactions: water. Yet, in the membrane, there are infinite possibilities of chemical compositions, and the physical properties of this diverse solvent are not a single set point but a sliding scale. The primary role of the lipid bilayer is to provide sufficient electrical and chemical barriers while maintaining fluidity to allow membrane proteins to function (Deamer, 2017). It has been hypothesized that early biological membranes of the last universal common ancestor were formed from C_8 to C_{12} aliphatic acids before diverging into isoprene ethers in archaea and fatty acid esters in bacteria (Koonin and Martin, 2005). Typically, eukaryotic membranes are comprised of chain lengths from 16 to 18 carbons, consistent with the 3-nm hydrophobic thickness measured by Fricke, although there are some interesting biological examples of shorter membranes such as in organelles like the Golgi apparatus (Bretscher and Munro, 1993; Sharpe et al., 2010). There is considerable diversity among lipid head group composition across different organisms, as well as chemistry in the acyl chains, including differing amounts of saturation and branch chain modifications. Eukaryotic membranes can contain high amounts of cholesterol. Furthermore, archaeal membranes are sometimes found to exist not as bilayers but as monolayers of tetraether lipids that resemble two phospholipids covalently connected at their tails (Valentine, 2007). To top it all off, biological membranes are often asymmetric across the inner and outer leaflets. Yet, despite all of this complexity, it is apparent that membrane proteins from the same family are homologous in structure despite striking differences in the native solvent environments. The long-held interpretation of this was that the lipids act mainly as inert nonspecific solvent molecules. Yet, we know that lipids play an important role in regulating function and protein stability. With this, it appears that many essential properties of membrane proteins are robust and can withstand small changes in lipid composition, but the reactions of these membrane proteins can be finely tuned by changes within the lipid bilayer.

An important consequence of a biologically realistic, mixed lipid bilayer is the tendency for these systems to demonstrate phase separation. In the 1990s, membrane fragments were identified 10–200 nm in size, rich in cholesterol and sphingolipids as well as membrane proteins (Simons and Ikonen, 1997). These fragments were called "rafts," proposed to be self-assembled domains that were important in regulating membrane signaling and function. Because of the challenges in imaging such small structures at the time, the study of rafts has been under ongoing debate. Yet, there is substantial evidence that mixed lipid systems exhibit immiscible liquid phase separation and critical point behavior (Veatch et al., 2007; Honerkamp-Smith et al., 2009; Sezgin et al., 2017). Furthermore, it is apparent that receptors and membrane lipids may cluster and aggregate together and that this may affect physiological signaling. Therefore, mixed membranes offer a way of regulating membrane physiology beyond the activity of each individual protein, and this can be modulated by changes in lipid synthesis or metabolism (Siliakus et al., 2017). As we learn that lipids have a significant and intimate relationship with membrane proteins, it becomes clear that we require a quantitative understanding of how membrane proteins physically behave in more complex membrane systems. This requires multiple pathways of research studying membrane protein structure and function in membranes, the physical properties of these complex lipid bilayers by methods such as small angle x-ray or neutron scattering, and superresolution microscopy imaging. Although complicated, there is an enormous amount of information to learn that is sure to have an effect on the complexities of cell membrane physiology.

Conclusions

Cell membrane research over the last century has taken us from invisible boundaries to a structural lipid bilayer—a low dielectric electrostatic barrier that is both fluid and flexible. Embedded within the membrane exists a completely different type of protein: membrane proteins that are hydrophobic in nature and present energetically favorable solutions for the passage of ions

and molecules through selective pores or transport pathways. Now, with all of this structural information in place, we are faced with the question of how these proteins physically behave inside the lipid bilayer. The reactions of membrane proteins, from folding, binding, and conformational exchange, occur in this unique solvent environment that is essentially a layer of organized oil. It is so distinctly different from water and has a diverse chemical composition that changes from organelle to cell type to organism. Future research will need to account for the inherent complexities and diversity of membrane systems when working toward a quantitative understanding of membrane protein behavior in membranes. This is an important goal toward a thorough understanding of how the membrane and its protein constituents define the physiology of each living cell.

Acknowledgments

References

Andersen, O.S., and R.E. Koeppe II. 2007. Bilayer thickness and membrane protein function: an energetic perspective. *Annu. Rev. Biophys. Biomol. Struct.* 36:107–130. https://doi.org/10.1146/annurev.biophys.36.040306.132643

Anselmi, C., K.M. Davies, and J.D. Faraldo-Gómez. 2018. --Mitochondrial ATP synthase dimers spontaneously associate due to a long-range membrane-induced force. *J. Gen. Physiol.* 150:763–770. https://doi.org/10.1085/jgp.201812033

Bar, R.S., D.W. Deamer, and D.G. Cornwell. 1966. Surface area of human erythrocyte lipids: reinvestigation of experiments on plasma membrane. *Science.* 153:1010–1012. https://doi.org/10.1126/science.153.3739.1010

Bean, R.C., W.C. Shepherd, H. Chan, and J. Eichner. 1969. Discrete conductance fluctuations in lipid bilayer protein membranes. *J. Gen. Physiol.* 53:741–757. https://doi.org/10.1085/jgp.53.6.741

Bretscher, M.S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science.* 261:1280–1281. https://doi.org/10.1126/science.8362242

Caffrey, M., and G.W. Feigenson. 1981. Fluorescence quenching in model membranes. 3. Relationship between calcium adenosinetriphosphatase enzyme activity and the affinity of the protein for phosphatidylcholines with different acyl chain characteristics. *Biochemistry.* 20:1949–1961. https://doi.org/10.1021/bi00510a034

Campagna, J.A., K.W. Miller, and S.A. Forman. 2003. Mechanisms of actions of inhaled anesthetics. *N. Engl. J. Med.* 348:2110–2124. https://doi.org/10.1056/NEJMra021261

Chadda, R., V. Krishnamani, K. Mersch, J. Wong, M. Brimberry, A. Chadda, L. Kolmakova-Partensky, L.J. Friedman, J. Gelles, and J.L. Robertson. 2016. The dimerization equilibrium of a ClC Cl⁻/H⁺ antiporter in lipid bilayers. *eLife.* 5:e17438. https://doi.org/10.7554/eLife.17438

Chadda, R., L. Cliff, M. Brimberry, and J.L. Robertson. 2018. A model-free method for measuring dimerization free energies of CLC-ec1 in lipid bilayers. *J. Gen. Physiol.* 150:355–365. https://doi.org/10.1085/jgp.201711893

Cristian, L., J.D. Lear, and W.F. DeGrado. 2003. Use of thiol-disulfide equilibria to measure the energetics of assembly of transmembrane helices in phospholipid bilayers. *Proc. Natl. Acad. Sci. USA.* 100:14772–14777. https://doi.org/10.1073/pnas.2536751100

Danielli, J.F., and H. Davson. 1935. A contribution to the theory of permeability of thin films. *J. Cell. Comp. Physiol.* 5:495–508. https://doi.org/10.1002/jcp.1030050409

Deamer, D. 2017. The role of lipid membranes in life's origin. *Life (Basel).* 7:E5. https://doi.org/10.3390/life7010005

Dean, R.B. 1941. Theories of electrolyte equilibrium in muscle. *Biol. Symp.* 3:331–348.

Deisenhofer, J., O. Epp, K. Miki, R. Huber, and H. Michel. 1985. Structure of the protein subunits in the photosynthetic reaction centre of Rhodopseudomonas viridis at 3Å resolution. *Nature.* 318:618–624. https://doi.org/10.1038/318618a0

de Jesus, A.J., and T.W. Allen. 2013. The determinants of hydrophobic mismatch response for transmembrane helices. *Biochim. Biophys. Acta.* 1828:851–863. https://doi.org/10.1016/j.bbamem.2012.09.012

Doyle, D.A., J. Morais Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K⁺ conduction and selectivity. *Science.* 280:69–77. https://doi.org/10.1126/science.280.5360.69

Eriksson, U.K., G. Fischer, R. Friemann, G. Enkavi, E. Tajkhorshid, and R. Neutze. 2013. Subangstrom resolution X-ray structure details aquaporin-water interactions. *Science.* 340:1346–1349. https://doi.org/10.1126/science.1234306

Finkelstein, A. 1976. Water and nonelectrolyte permeability of lipid bilayer membranes. *J. Gen. Physiol.* 68:127–135. https://doi.org/10.1085/jgp.68.2.127

Fricke, H. 1925. The electrical capacity of suspensions with special reference to blood. *J. Gen. Physiol.* 9:137–152. https://doi.org/10.1085/jgp.9.2.137

Fu, H., Y. Liu, F. Adrià, X. Shao, W. Cai, and C. Chipot. 2014. From material science to avant-garde cuisine. The art of shaping liquids into spheres. *J. Phys. Chem. B.* 118:11747–11756. https://doi.org/10.1021/jp508841p

Gonen, T., Y. Cheng, P. Sliz, Y. Hiroaki, Y. Fujiyoshi, S.C. Harrison, and T. Walz. 2005. Lipid-protein interactions in double-layered two-dimensional AQP0 crystals. *Nature.* 438:633–638. https://doi.org/10.1038/nature04321

Goodsell, D. 2010. Molecule of the month. https://doi.org/10.2210/rcsb_pdb/mom_2010_1

Gorter, E., and F. Grendel. 1925. On bimolecular layers of lipoids on the chromocytes of the blood. *J. Exp. Med.* 41:439–443. https://doi.org/10.1084/jem.41.4.439

Gupta, K., J.A.C. Donlan, J.T.S. Hopper, P. Uzdavinys, M. Landreh, W.B. Struwe, D. Drew, A.J. Baldwin, P.J. Stansfeld, and C.V. Robinson. 2017. The role of interfacial lipids in stabilizing membrane protein oligomers. *Nature.* 541:421–424. https://doi.org/10.1038/nature20820

Helenius, A., and K. Simons. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta.* 415:29–79. https://doi.org/10.1016/0304-4157(75)90016-7

Henderson, R., and P.N. Unwin. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature.* 257:28–32. https://doi.org/10.1038/257028a0

Hendrickson, W.A. 2016. Atomic-level analysis of membrane-protein structure. *Nat. Struct. Mol. Biol.* 23:464–467. https://doi.org/10.1038/nsmb.3215

Hessa, T., H. Kim, K. Bihlmaier, C. Lundin, J. Boekel, H. Andersson, I. Nilsson, S.H. White, and G. von Heijne. 2005. Recognition of transmembrane helices by the endoplasmic reticulum translocon. *Nature.* 433:377–381. https://doi.org/10.1038/nature03216

Hilgemann, D.W., G. Dai, A. Collins, V. Larricia, S. Magi, C. Deisl, and M. Fine. 2018. Lipid signaling to membrane proteins: From second messengers to membrane domains and adapter-free endocytosis. *J. Gen. Physiol.* 150:211–224. https://doi.org/10.1085/jgp.201711875

Hille, B. 2018. The founding of *Journal of General Physiology*: Membrane permeation and ion selectivity. *J. Gen. Physiol.* 150:389–400. https://doi.org/10.1085/jgp.201711937

Hite, R.K., Z. Li, and T. Walz. 2010. Principles of membrane protein interactions with annular lipids deduced from aquaporin-0 2D crystals. *EMBO J.* 29:1652–1658. https://doi.org/10.1038/emboj.2010.68

Hladky, S.B., and D.A. Haydon. 1970. Discreteness of conductance change in bimolecular lipid membranes in the presence of certain antibiotics. *Nature.* 225:451–453. https://doi.org/10.1038/225451a0

Honerkamp-Smith, A.R., S.L. Veatch, and S.L. Keller. 2009. An introduction to critical points for biophysicists; observations of compositional heterogeneity in lipid membranes. *Biochim. Biophys. Acta.* 1788:53–63. https://doi.org/10.1016/j.bbamem.2008.09.010

Hong, H., T.M. Blois, Z. Cao, and J.U. Bowie. 2010. Method to measure strong protein-protein interactions in lipid bilayers using a steric trap. *Proc. Natl. Acad. Sci. USA.* 107:19802–19807. https://doi.org/10.1073/pnas.1010348107

Hooke, R. 1665. Micrographia. J. Martyn and J. Allestry, London. https://doi.org/https://doi.org/10.5962/bhl.title.904

Kleinzeller, A. 1997. Ernest Overton's contribution to the cell membrane concept: A centennial appreciation. *Physiology (Bethesda)*. 12:49–53. https://doi.org/10.1152/physiologyonline.1997.12.1.49

Kleinzeller, A. 1999. Charles Ernest Overton's concept of a cell membrane. *In* Membrane permeability, 100 years since Ernest Overton. Vol. 48. D.W. Deamer, A. Kleinzeller, and D.M. Fambrough, editors. Elsevier, New York. 1–22., https://doi.org/10.1016/S0070-2161(08)61039-4

Koonin, E.V., and W. Martin. 2005. On the origin of genomes and cells within inorganic compartments. *Trends Genet.* 21:647–654. https://doi.org/10.1016/j.tig.2005.09.006

Kučerka, N., M.-P. Nieh, J. Pencer, J.N. Sachs, and J. Katsaras. 2009. What determines the thickness of a biological membrane. *Gen. Physiol. Biophys.* 28:117–125. https://doi.org/10.4149/gpb_2009_02_117

Laganowsky, A., E. Reading, T.M. Allison, M.B. Ulmschneider, M.T. Degiacomi, A.J. Baldwin, and C.V. Robinson. 2014. Membrane proteins bind lipids selectively to modulate their structure and function. *Nature.* 510:172–175. https://doi.org/10.1038/nature13419

Langmuir, I. 1917. The constitution and fundamental properties of solids and liquids. II. Liquids. *J. Am. Chem. Soc.* 39:1848–1906. https://doi.org/10.1021/ja02254a006

Lee, A.G. 2003. Lipid-protein interactions in biological membranes: a structural perspective. *Biochim. Biophys. Acta.* 1612:1–40. https://doi.org/10.1016/S0005-2736(03)00056-7

Levine, Y.K., and M.H. Wilkins. 1971. Structure of oriented lipid bilayers. *Nat. New Biol.* 230:69–72. https://doi.org/10.1038/newbio230069a0

Lewis, B.A., and D.M. Engelman. 1983. Bacteriorhodopsin remains dispersed in fluid phospholipid bilayers over a wide range of bilayer thicknesses. *J. Mol. Biol.* 166:203–210. https://doi.org/10.1016/S0022-2836(83)80006-0

Loeb, J. 1912. Antagonistic action of electrolytes and permeability of the cell membrane. *Science.* 36:637–639. https://doi.org/10.1126/science.36.932.637-a

Lombard, J. 2014. Once upon a time the cell membranes: 175 years of cell boundary research. *Biol. Direct.* 9:32. https://doi.org/10.1186/s13062-014-0032-7

Lundbaek, J.A., P. Birn, A.J. Hansen, R. Søgaard, C. Nielsen, J. Girshman, M.J. Bruno, S.E. Tape, J. Egebjerg, D.V. Greathouse, et al. 2004. Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of Micelle-forming amphiphiles and cholesterol. *J. Gen. Physiol.* 123:599–621. https://doi.org/10.1085/jgp.200308996

Meyer, H. 1899. Zur theorie der alkoholnarkose. I. Mitt. *Arch. Exp. Pathol. Pharmakol.* 42:109–118. https://doi.org/10.1007/BF01834479

Miller, C., and E. Racker. 1979. Reconstitution of membrane transport functions. *In* General Principles and Procedures. Springer, Boston. 1–31. https://doi.org/https://doi.org/10.1007/978-1-4684-0979-6_1

Min, D., R.E. Jefferson, J.U. Bowie, and T.Y. Yoon. 2015. Mapping the energy landscape for second-stage folding of a single membrane protein. *Nat. Chem. Biol.* 11:981–987. https://doi.org/10.1038/nchembio.1939

Min, D., R.E. Jefferson, Y. Qi, J.Y. Wang, M.A. Arbing, W. Im, and J.U. Bowie. 2018. Unfolding of a ClC chloride transporter retains memory of its evolutionary history. *Nat. Chem. Biol.* 14:489–496. https://doi.org/10.1038/s41589-018-0025-4

Mitchell, P. 1957. A general theory of membrane transport from studies of bacteria. *Nature.* 180:134–136. https://doi.org/10.1038/180134a0

Moon, C.P., and K.G. Fleming. 2011. Side-chain hydrophobicity scale derived from transmembrane protein folding into lipid bilayers. *Proc. Natl. Acad. Sci. USA.* 108:10174–10177. https://doi.org/10.1073/pnas.1103979108

Moor, H., and K. Mühlethaler. 1963. Fine structure in frozen-etched yeast cells. *J. Cell Biol.* 17:609–628. https://doi.org/10.1083/jcb.17.3.609

Mouritsen, O.G., and M. Bloom. 1984. Mattress model of lipid-protein interactions in membranes. *Biophys. J.* 46:141–153. https://doi.org/10.1016/S0006-3495(84)84007-2

Mueller, P., D.O. Rudin, H.T. Tien, and W.C. Wescott. 1962. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature.* 194:979–980. https://doi.org/10.1038/194979a0

Norimatsu, Y., K. Hasegawa, N. Shimizu, and C. Toyoshima. 2017. Protein-phospholipid interplay revealed with crystals of a calcium pump. *Nature.* 545:193–198. https://doi.org/10.1038/nature22357

Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of Halobacterium halobium. *Nat. New Biol.* 233:149–152. https://doi.org/10.1038/newbio233149a0

Osterhout, W.J.V. 1911. The permeability of living cells to salts in pure and balanced solutions. *Science.* 34:187–189. https://doi.org/10.1126/science.34.867.187

Overton, C.E. 1895. Über die osmotischen Eigenschaften der lebenden Pflanzen-und Tierzelle. Vierteljahrschr. Naturforsch. Ges. Zürich. 40:159–201.

Overton, C.E. 1901. Studien über die Narkose, zugleich ein Beitrag zur allgemeinen Pharmakologie. Gustav Fischer, Jena.

Parsegian, A. 1969. Energy of an ion crossing a low dielectric membrane: solutions to four relevant electrostatic problems. *Nature.* 221:844–846. https://doi.org/10.1038/221844a0

Pfeffer, W. 1877. Osmotische Untersuchungen. Engelmann, Leipzig.

Pockels, A. 1892. On the relative contamination of the water-surface by equal quantities of different substances. *Nature.* 46:418–419. https://doi.org/10.1038/046418e0

Popot, J.L., and D.M. Engelman. 1990. Membrane protein folding and oligomerization: the two-stage model. *Biochemistry.* 29:4031–4037. https://doi.org/10.1021/bi00469a001

Popot, J.L., S.E. Gerchman, and D.M. Engelman. 1987. Refolding of bacteriorhodopsin in lipid bilayers. A thermodynamically controlled two-stage process. *J. Mol. Biol.* 198:655–676. https://doi.org/10.1016/0022-2836(87)90208-7

Rayleigh, L. 1889. Measurements of the amount of oil necessary in order to check the motions of camphor upon water. *Proc. R. Soc. Lond.* 47:364–367. https://doi.org/10.1098/rspl.1889.0099

Richardson, S.H., H.O. Hultin, and D.E. Green. 1963. Structural proteins of membrane systems. *Proc. Natl. Acad. Sci. USA.* 50:821–827. https://doi.org/10.1073/pnas.50.5.821

Riederer, E.A., P.J. Focke, E.R. Georgieva, N. Akyuz, K. Matulef, P.P. Borbat, J.H. Freed, S.C. Blanchard, O. Boudker, and F.I. Valiyaveetil. 2018. A facile approach for the in vitro assembly of multimeric membrane transport proteins. *eLife.* 7:e36478. https://doi.org/10.7554/eLife.36478

Roberts, G. 2013. Langmuir-Blodgett Films. Springer Science and Business Media, Boston. https://doi.org/10.1007/978-1-4899-3716-2

Robertson, J.D. 1959. The ultrastructure of cell membranes and their derivatives. *Biochem. Soc. Symp.* 16:3–43.

Robertson, J.D. 1981. Membrane structure. *J. Cell Biol.* 91:189s–204s. https://doi.org/10.1083/jcb.91.3.189s

Sanders, M.R., H.E. Findlay, and P.J. Booth. 2018. Lipid bilayer composition modulates the unfolding free energy of a knotted α-helical membrane protein. *Proc. Natl. Acad. Sci. USA.* 115:E1799–E1808. https://doi.org/10.1073/pnas.1714668115

Schrum, J.P., T.F. Zhu, and J.W. Szostak. 2010. The origins of cellular life. *Cold Spring Harb. Perspect. Biol.* 2:a002212. https://doi.org/10.1101/cshperspect.a002212

Sezgin, E., I. Levental, S. Mayor, and C. Eggeling. 2017. The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat. Rev. Mol. Cell Biol.* 18:361–374. https://doi.org/10.1038/nrm.2017.16

Sharpe, H.J., T.J. Stevens, and S. Munro. 2010. A comprehensive comparison of transmembrane domains reveals organelle-specific properties. *Cell.* 142:158–169. https://doi.org/10.1016/j.cell.2010.05.037

Siliakus, M.F., J. van der Oost, and S.W.M. Kengen. 2017. Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. *Extremophiles.* 21:651–670. https://doi.org/10.1007/s00792-017-0939-x

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* 387:569–572. https://doi.org/10.1038/42408

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science.* 175:720–731. https://doi.org/10.1126/science.175.4023.720

Skou, J.C. 1998. Nobel Lecture. The identification of the sodium pump. *Biosci. Rep.* 18:155–169. https://doi.org/10.1023/A:1020196612909

Stillwell, W. 2016. An Introduction to Biological Membranes. Elsevier, London.

Stoeckenius, W., and R. Rowen. 1967. A morphological study of Halobacterium halobium and its lysis in media of low salt concentration. *J. Cell Biol.* 34:365–393. https://doi.org/10.1083/jcb.34.1.365

Tanford, C. 2004. Ben Franklin Stilled the Waves. Oxford University Press, Oxford. https://doi.org/https://doi.org/10.1177/027046769001000233

Tanford, C., and J.A. Reynolds. 1976. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta.* 457:133–170. https://doi.org/10.1016/0304-4157(76)90009-5

Tien, H.T. 1968. Black lipid membranes at bifaces. Formation characteristics, optical and some thermodynamic properties. *J. Gen. Physiol.* 52(1, Suppl–43s):43s. https://doi.org/10.1085/jgp.52.1.125

Traube, M. 1867. Experimente zur Theorie der Zellenbildung und Endosmose. *Archiv. Anat. Physiol.* 87–165.

Urry, D.W., T.L. Trapane, and K.U. Prasad. 1983. Is the gramicidin a transmembrane channel single-stranded or double-stranded helix? A simple

unequivocal determination. *Science.* 221:1064–1067. https://doi.org/10.1126/science.221.4615.1064

Valentine, D.L. 2007. Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat. Rev. Microbiol.* 5:316–323. https://doi.org/10.1038/nrmicro1619

Valiyaveetil, F.I., Y. Zhou, and R. MacKinnon. 2002. Lipids in the structure, folding, and function of the KcsA K+ channel. *Biochemistry.* 41:10771–10777. https://doi.org/10.1021/bi026215y

Veatch, W., and L. Stryer. 1977. The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. *J. Mol. Biol.* 113:89–102. https://doi.org/10.1016/0022-2836(77)90042-0

Veatch, S.L., O. Soubias, S.L. Keller, and K. Gawrisch. 2007. Critical fluctuations in domain-forming lipid mixtures. *Proc. Natl. Acad. Sci. USA.* 104:17650–17655. https://doi.org/10.1073/pnas.0703513104

von Nägeli, C.W., and C.E. Cramer. 1855. Pflanzenphysiologische Untersuchungen. Bel Friedrich Schluthess, Zürich.

Weiss, M.S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G.E. Schulz. 1991. Molecular architecture and electrostatic properties of a bacterial porin. *Science.* 254:1627–1630. https://doi.org/10.1126/science.1721242

White, S.H., and W.C. Wimley. 1999. Membrane protein folding and stability: physical principles. *Annu. Rev. Biophys. Biomol. Struct.* 28:319–365. https://doi.org/10.1146/annurev.biophys.28.1.319

Wilkins, M.H., A.E. Blaurock, and D.M. Engelman. 1971. Bilayer structure in membranes. *Nat. New Biol.* 230:72–76. https://doi.org/10.1038/newbio230072a0

Wimley, W.C., and S.H. White. 2000. Designing transmembrane alpha-helices that insert spontaneously. *Biochemistry.* 39:4432–4442. https://doi.org/10.1021/bi992746j

Wimley, W.C., T.P. Creamer, and S.H. White. 1996. Solvation energies of amino acid side chains and backbone in a family of host-guest pentapeptides. *Biochemistry.* 35:5109–5124. https://doi.org/10.1021/bi9600153

Yu, H., M.G.W. Siewny, D.T. Edwards, A.W. Sanders, and T.T. Perkins. 2017. Hidden dynamics in the unfolding of individual bacteriorhodopsin proteins. *Science.* 355:945–950. https://doi.org/10.1126/science.aah7124