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INTRODUCTION

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1.1 HISTORY

Modern membrane science can be traced back to 1748 to the work of the French priest and physicist Jean-Antoine (Abbé) Nollet who, in the course of an experiment in which he immersed a pig's bladder containing alcohol in water, accidentally discovered the phenomenon of osmosis [1], that is, the movement of water across a semipermeable membrane. The term osmosis was, however, first introduced [2] by another French scientist, Henri Dutrochet, in 1827. The movement of water across a membrane in osmosis is a passive diffusion process driven by the difference in chemical potential (or activity) of water on each side of the membrane. The diffusion of water can be through the predominant matrix of which the membrane is composed, that is, lipid in the case of biological membranes, or through proteins incorporated in the membrane, for example, aquaporins. As the title of this book suggests, here we limit ourselves to a discussion of the movement of ions and metabolites through membranes via proteins embedded in them, rather than of transport through the lipid matrix of biological membranes.

The fact that small ions, in particular Na^+, K^+, and Cl^-, are not evenly distributed across the plasma membrane of cells (see Fig. 1.1) was first recognized by the physiological chemist Carl Schmidt [3] in the early 1850s. Schmidt was investigating the pathology of cholera, which was widespread in his native Russia at the time, and

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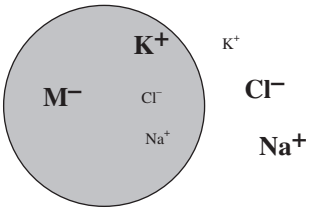

FIGURE 1.1 Ionic distributions across animal cell membranes. M^- represents impermeant anions, for example, negatively charged proteins. Typical intracellular (int) and extracellular (ext) concentrations of the small inorganic ions are: $[K^+]_{int} = 140–155\,mM$, $[K^+]_{ext} = 4–5\,mM$, $[Cl^-]_{int} = 4\,mM$, $[Cl^-]_{ext} = 120\,mM$, $[Na^+]_{int} = 12\,mM$, $[Na^+]_{ext} = 145–150\,mM$. (Note: In the special case of red blood cells $[Cl^-]_{ext}$ is lower (98–109 mM) due to exchange with HCO_3^- across the plasma membrane, which is important for CO_2 excretion and the maintenance of blood pH. This exchange is known as the "chloride shift.") Adapted from Ref. 4 with permission from Wiley.

discovered the differences in ion concentrations while comparing the blood from cholera victims and healthy individuals. By the end of the nineteenth century it was clear that these differences in ionic distributions occurred not only in blood, but existed across the plasma membrane of cells from all animal tissues. However, the origin of the concentration differences remained controversial for many years.

In the 1890s at least two scientists, Rudolf Heidenhain [5] (University of Breslau) and Ernest Overton [6] (then at the University of Zürich), both reached the conclusion that the Na^+ concentration gradient across the membrane was produced by a pump, situated in the cell membrane, which derived its energy from metabolism. Although we know now that this conclusion is entirely correct, it was apparently too far ahead of its time. In 1902, Overton even correctly proposed [7] that an exchange of Na^+ and K^+ ions across the cell membrane of muscle—now known to arise from the opening and closing of voltage-sensitive Na^+ and K^+ channels—was the origin of the change in electrical voltage leading to muscle contraction. This proposal too was not widely accepted at the time or even totally ignored. It took another 50 years before Overton's hypothesis was rediscovered and finally verified by the work of Hodgkin and Huxley [8], for which they both received the Nobel Prize in Physiology or Medicine in 1963. According to Kleinzeller [9], Andrew Huxley once said that, "If people had listened to what Overton had to say about excitability, the work of Alan [Hodgkin] and myself would have been obsolete."

Unfortunately for Heidenhain and Overton, their work did not conform with the *Zeitgeist* of the early twentieth century. At the time, much fundamental work on the theory of diffusion was being carried out by high-profile physicists and physical chemists, among them van't Hoff, Einstein, Planck, and Nernst. Of particular relevance for the distribution of ions across the cell membranes was the work of the Irish physical chemist Frederick Donnan [10] on the effect of nondialyzable salts. Therefore, it was natural that physiologists of the period would try to explain membrane transport in terms of passive diffusion alone, rather than adopt Overton's and Heidenhain's controversial hypothesis of ion pumping or active transport.

Donnan [10] suggested that if the cytoplasm of cells contained electrolytically dissociated nondialyzable salts (e.g., protein anions), which it does, small permeable ions would distribute themselves across the membrane so as to maintain electroneutrality in both the cytoplasm and the extracellular medium. Thus, the cytoplasm would naturally tend to attract small cations, whereas the extracellular medium would accumulate anions. Referring back to Figure 1.1, one can see that this idea could explain the distribution of K^+ and Cl^- ions across the cell membrane. However, the problem is that the so-called Donnan equilibrium doesn't explain the distribution of Na^+ ions. Based on Donnan's theory, any permeable ion of the same charge should adopt the same distribution across the membrane, but the distributions of Na^+ and K^+ are in fact the opposite of one another. To find a rational explanation for this inconsistency, many physiologists concluded that, whereas cell membranes were permeable to K^+ and Cl^- ions, they must be completely impermeable to Na^+ ions. The logical consequence of this was that the Na^+ concentration gradient should have originated at the first stages of the cell division and persisted throughout each animal's entire life. This view was an accepted doctrine for the next 30 years following the publication of Donnan's theory [11].

In the late 1930s and early 1940s, however, evidence was mounting that the idea of an impermeant Na^+ ion was untenable. In this period, radioisotopes started to become available for research, which greatly increased the accuracy of ion transport measurements. A further stimulus at the time was the development in the United States of blood banks and techniques for blood transfusion, during which researchers were again investigating the distribution of ions across the red blood cell membranes and the effects of cold storage. Researchers in the United States, in particular at the University of Rochester, Yale University, and the State University of Iowa, were now the major players in the field, among them Fenn, Heppel, Steinbach, Peters, Danowski, Harris, and Dean. Details of the experimental evidence that led to the universal discarding of the notion of an impermeant Na^+ ion and the reemergence of the hypothesis of an active Na^+ pump located in the cell membrane of both excitable and nonexcitable cells are described elsewhere [4, 12, 13]. Here it suffices to say that by the middle of the twentieth century the active transport of Na^+ had become an established fact.

The enzyme responsible for active Na^+ transport, the Na^+,K^+-ATPase, which is powered by the energy released from ATP hydrolysis, was isolated by Jens Christian Skou of the University of Aarhus, Denmark, in 1957 [14]. This was the first ever ion-transporting enzyme to be identified. Almost 40 years later, in 1997, when all possible doubt that Skou's Na^+,K^+-ATPase incorporated the complete active transport machinery for sodium and potassium ions and the broad significance of his discovery was clear, he received the Nobel Prize in Chemistry.

One reason why the concept of the active transport of Na^+ took so long to be accepted was probably the perception that it represented a waste of a cell's valuable energy resources. However, rather than think of the pumping of ions across a membrane as energy expenditure, in fact it is more helpful and more accurate to describe it as an energy conversion process. In the case of the Na^+,K^+-ATPase, the energy released by ATP hydrolysis is stored as Na^+ and K^+

electrochemical potential gradients across the membrane. Therefore, the energy can be released again whenever Na^+ or K^+ diffuse passively across the membrane. The Na^+ and K^+ electrochemical potential gradients established by the Na^+,K^+-ATPase across the plasma membrane of all animal cells thus provide the driving force for diffusion of Na^+ and K^+ through all plasma membrane Na^+- and K^+-selective ion channels, which, for example, is the basis of the production of action potentials in nerve and muscle. The Na^+ electrochemical potential gradient created by the Na^+,K^+-ATPase also serves as a secondary source of energy to drive the active uptake or extrusion of other ions or metabolites across the plasma membrane by transporter membrane proteins. For example, the reabsorption of glucose into the bloodstream in the kidney is driven by the energy released by the simultaneous coupled passive flow of Na^+ into the cytoplasm of the epithelial cells lining the kidney collecting tubules. As these examples demonstrate, the realization that the cell membrane is permeable to Na^+ ions and requires a sodium pump to keep the ions out was pivotal for the understanding of membrane transport processes in general, and the change in thinking that this realization generated no doubt contributed to the later discovery of many other membrane protein transport systems, including channels and transporters.

Now we will concentrate for the moment on channels alone. When Hodgkin and Huxley proposed [8] consecutive changes in Na^+ and K^+ membrane permeability of nerve as the origin of the action potential in 1952, their hypothesis was based on the mathematical fitting of kinetic equations to their recorded data. Impressive as their conclusions were, their data still provided no clue as to the molecular origin of the changes in Na^+ or K^+ permeability. A major step forward occurred in 1964 when Narahashi et al. [15] discovered that tetrodotoxin (TTX), a paralytic poison found in some edible (with caution) puffer fish, blocks the action potential in nerve axons by inhibiting the Na^+ conductance but without any effect on the K^+ conductance. This clearly demonstrated that there must be separate pathways or channels for Na^+ and K^+ ions in the membrane. Still the chemical nature of the channels was unclear, but after this discovery TTX became an invaluable tool for the identification of the source of the Na^+ conductance.

The next major advance in the channel field occurred through the application of biochemical purification procedures. The electric eel, *Electrophorus electricus*, is capable of producing voltages as high as 600 V along the whole animal. As one might imagine, its specialized electrical properties made it a prime source for the isolation of the molecules responsible for voltage changes across the cell membranes. In 1978 Agnew et al. [16] succeeded in extracting and purifying a 230 kDa protein that had a high affinity for TTX. After it was shown in 1984 [17] that synthetic vesicles, in which the purified protein had been reconstituted, displayed Na^+ currents that could be inhibited by TTX, there was no longer any doubt that the Na^+ channel had been isolated and that it was indeed a membrane protein. More details on the history of ion channel research including more recent developments can be found in a fine review by Bezanilla [18].

Now, finally in this brief historical overview, we turn our attention to transporters. Particularly in the intestine and in the kidney, many metabolites, including sugars

and amino acids, need to be absorbed or reabsorbed, respectively, into the bloodstream. In the early 1960s, shortly after Skou's discovery of the Na^+,K^+-ATPase [14], Robert Crane first suggested [19, 20] that the intestinal absorption of sugar was coupled to the influx of Na^+ into the cell, that is, that the energy released by the passive diffusion of Na^+ into the cell was utilized to absorb sugars. His hypothesis was based in part on the fact that sugar absorption was already known to be dependent on the presence of Na^+ in the medium. Roughly 10 years later, using isolated intestinal epithelial cells, Kimmich [21] showed the sugar uptake system was located in the plasma membrane of the cells and not between the cells of an intact tissue or epithelium. That the Na^+/glucose coupled transport system is in fact a membrane protein was shown in a similar way to that described above for the Na^+ channel, that is, by isolation of the protein from tissue, reconstitution in vesicles, and the demonstration that the reconstituted system carried out Na^+-dependent active transport of glucose across the vesicle membrane [22]. For these experiments, kidney tissue was used because of the higher concentration of the protein that could be isolated in comparison to intestine.

A useful question to ask here is why such coupled transport systems, at least in animals, all utilize the Na^+ gradient across the membrane and not the K^+ gradient. The answer is quite simple. The distribution of K^+ ions across the plasma membrane is quite close to equilibrium, that is, the normal resting electrical potential across the membrane is quite close to what one would calculate theoretically based on the equilibrium theory of Nernst for electrical diffusion potentials. In contrast, the distribution of Na^+ is far from equilibrium. Therefore, the passive diffusion of Na^+ in through a transporter protein releases much more energy that can be used for metabolite uptake or extrusion than the passive diffusion of K^+ out.

At roughly the same time that Crane hypothesized the coupling of the energy stored in the Na^+ gradient to glucose absorption, the idea of energy storage in electrochemical potential gradients was also taken up by Peter Mitchell [23] when he proposed the chemiosmotic theory of oxidative and photosynthetic phosphorylation, for which he received the 1978 Nobel Prize in Chemistry. Central to Mitchell's hypothesis was the existence of a membrane-bound ATPase in mitochondria or chloroplasts that utilized the H^+ gradient built up across their inner membranes for the conversion of ADP to ATP. This enzyme, now known as the ATP synthase or F_0F_1-ATPase, cannot strictly be classified as a transporter, because the energy released as H^+ ions that flows through it across the membrane is not used for the transport of other ions or metabolites, but rather it is converted into chemical energy in the form of ATP. Closely related molecular machines are the bacterial flagellar motors, which also use the energy of an H^+ gradient, but in this case the energy is released in mechanical form as flagellar rotation.

Concluding this historical overview, one can say that the existence of membrane-embedded proteins that act as pumps, channels, and transporters and the means by which they gain their energy to carry out their transport processes were firmly established by the early 1980s. Since that time, further major advances have been made into the details of how they operate. One significant advance was the development of patch-clamp techniques by Neher and Sakmann [24], which enabled the opening and closing of single

channels to be directly recorded, and for which they received the Nobel Prize for Physiology or Medicine in 1991. Another major advance has been the resolution of the atomic structure of membrane proteins by X-ray crystallography. The first membrane protein to be crystallized and have its structure determined by X-ray diffraction was that of a bacterial photosynthetic reaction center [25], for which Michel, Deisenhofer, and Huber received the Nobel Prize in Chemistry in 1988. After a slow start, the structures of other membrane proteins at atomic resolution are now being determined at an increasingly rapid rate. With structures becoming available, this has allowed the application of molecular dynamics simulations and other theoretical techniques to obtain an improved chemical understanding of how pumps, channels, and transporters work. Both patch-clamp techniques and molecular simulations are topics of later chapters of this book.

1.2 ENERGETICS OF TRANSPORT

How does one distinguish between pumps, channels, and transporters? The decisive criterion is whether or not energy is required for transport. However, to decide whether the transport of an ion requires energy or not, it is not sufficient to consider its concentration, c, on each side of the membrane. The electrical potential difference, V_m, across the membrane also contributes to the energetics of the process. Therefore, one needs to define the electrochemical potential difference, $\Delta\mu$, which for the transport of an ion into a cell is given by,

$$\Delta\mu = RT \ln \frac{c_{in}}{c_{out}} + zFV_m \tag{1.1}$$

and for the transport of an ion out of a cell is given by,

$$\Delta\mu = RT \ln \frac{c_{out}}{c_{in}} - zFV_m \tag{1.2}$$

In these equations, R is the ideal gas constant, T is the absolute temperature, z is the valence of the ion (e.g., +1 for Na^+, or +2 for Ca^{2+}), F is Faraday's constant, and V_m is the electrical potential difference across the membrane. In both equations V_m is defined as the potential inside the cell minus the potential outside the cell. The movement of an ion across a membrane for which $\Delta\mu$ is calculated to be negative involves a loss of free energy. If the movement is along an electrochemical potential gradient, it is a spontaneous process, and no energy is required. If $\Delta\mu$ is calculated to be positive, on the other hand, then the movement of the ion requires energy, the movement is against an electrochemical potential gradient, the process is non-spontaneous, and a source of energy would be required. Of course, if it is an uncharged metabolite that is moving across the membrane then the second term in Equations 1.1 and 1.2 disappears, and it is only the direction of the chemical potential gradient or the concentration gradient that determines whether or not the transport is spontaneous.

If no energy is required, that is, the transport occurs spontaneously along an electrochemical potential gradient ($\Delta\mu < 0$), then the transport is termed *facilitated diffusion*. In this case, the protein simply provides a pathway for the ions to move more easily through the membrane. This is the situation that occurs with a channel. If energy is required, that is, the transport occurs nonspontaneously up an electrochemical potential gradient ($\Delta\mu > 0$), then the transport is termed *active transport*.

There are a number of possible sources of energy in the case of active transport. If the energy comes directly from light, ATP, or from the energy released in a redox reaction, then this is termed *primary active transport*. All pumps are primary active transporters. If the energy is generated by the flow of an ion down an electrochemical potential gradient created by a pump, then this is termed *secondary active transport*. The Na^+/glucose cotransporter is an example of this. In such a situation, the transport of one species is down an electrochemical potential gradient ($\Delta\mu < 0$) and the transport of the other is up an electrochemical potential gradient ($\Delta\mu > 0$). When summed together, as long as the overall $\Delta\mu$ is negative the transport proceeds. For example, in the case of the Na^+/glucose cotransporter, as long as $\Delta\mu(Na^+) + \Delta\mu(\text{glucose}) < 0$, then both glucose and Na^+ are taken up into the cytoplasm of the cell.

1.3 MECHANISTIC CONSIDERATIONS

Apart from the difference in energetics, there are also important mechanistic differences between active transport and facilitated diffusion processes. In active transport, because the ions are transported against an electrochemical potential gradient, the enzyme's ion-binding sites should not be open to both sides of the membrane simultaneously. If this were to happen the efficiency of pumping would be drastically compromised. The ions should first be bound from one side of the membrane, become occluded within the protein via a conformational change, and then be released to the other side of the membrane via a conformational change. This is in contrast to the mechanism of ion channels, which have their ion-binding sites open to both sides of the membrane at once (see Fig. 1.2). Because of these differences in mechanism, the transport timescales of ion pumps and transporters are very different from those of ion channels.

A channel that is open to both sides of the membrane at once allows a rapid flux of ions across the membrane. For example, the flux through open Na^+ channels of the nerve membrane is approximately 10^7 ions/s, corresponding to an average time for the transport of a single ion of 0.1 μs. In contrast, ion pumps and transporters function on a much slower timescale. In their case, ion transport requires significant conformational changes to drive the ions or metabolites across the membrane. Because these conformational changes involve a large number of amino acid side chains whose intermolecular interactions need to be broken and formed, they typically have rate constants on the order of $100\,s^{-1}$ or slower. The overall turnover of an ion pump or transporter then usually occurs on a timescale of milliseconds to seconds, that is, four to six orders of magnitude slower than that of ion channels. This has important experimental consequences. Because of the large ion fluxes that they produce, the

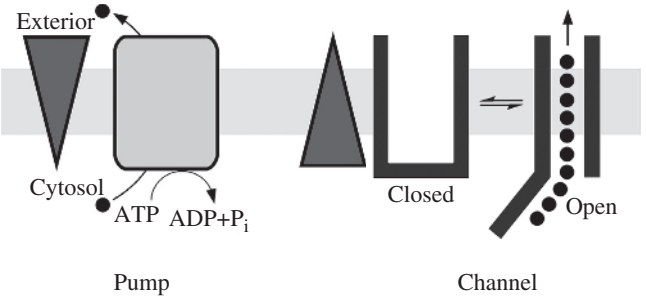

FIGURE 1.2 Ion-transporting membrane proteins. Channels can exist in an open state, in which ions move *down* an electrochemical potential gradient. No energy is required—the transport is termed facilitated diffusion. Pumps transport ions *against* an electrochemical potential gradient. The ion-binding sites are open alternately to the cytosol and the exterior. Energy is required—the transport is termed active transport. In the example shown the energy is derived from ATP hydrolysis. Reproduced from Ref. 26 with permission from Wiley.

opening and closing of single channels can be observed via the patch-clamp technique. Typically the observed currents are in the picoampere range. However, single ion pumps or transporters produce only very small currents across the membranes that are exceedingly difficult if not impossible to measure by electrophysiological means. An alternative approach for pumps is to use the whole-cell patch-clamp technique, whereby the ion flux through many pumps or transporters is recorded simultaneously.

The important point which we would like to make here is that because of these mechanistic and timescale differences, experimental techniques designed for the investigation of ion channels can often not be applied to pumps or transporters. In a similar fashion, techniques devised for research on pumps or transporters cannot generally be directly applied to ion channels. Therefore, in the following chapters one will find some experimental techniques that have been specifically designed with ion channel investigations in mind and others with ion pumps or transporters in mind.

1.4 ION CHANNELS

Ion channels can be classified according to what it is that causes them to open, that is, their gating mechanism. For a channel to allow ions to pass, it must undergo a conformational change from one or more inactive closed states. There are a number of mechanisms by which this conformational change might come about. Below we consider the variety of possible mechanisms one by one.

1.4.1 Voltage-Gated

First we consider voltage-gated channels, for example, the Na^+- and K^+-channels of nerve and muscle responsible for the action potential. If a channel contains movable charged or dipolar amino acid residues, then a change in voltage across the

membrane would cause a change in the electric field across the protein. The change in field strength could then induce a translational or rotational motion of the charged or dipolar regions of the protein leading to a conformational change that opens the channel. The charged or dipolar regions are termed the voltage sensor of the channel.

1.4.2 Ligand-Gated

Another important class of ion channels is those that are ligand-gated. In this case, the binding of a ligand to the channel induces a conformational change that leads to channel opening. Classic examples of this type of channel are the nicotinic acetyl choline receptors, which are located in the plasma membrane of nerve and muscle cells. Binding of the neurotransmitter acetyl choline to the receptor stimulates its channel activity allowing Na^+ and K^+ to flow through it. This causes an increase in the membrane potential (termed depolarization), which subsequently causes the opening of voltage-gated Na^+ channels, and the production of the action potential necessary for muscle contraction.

1.4.3 Mechanosensitive

Mechanosensitive channels are a further important class of ion channels. These respond to mechanical deformations of the membrane in which they are embedded, for example, changes in membrane tension, thickness, or curvature. They can be found in all forms of cellular life. An example is the stretch-activated large conductance mechanosensitive channels (MscL) of bacteria, which were first discovered in *Escherichia coli* by Martinac et al. [27] in 1987. These channels allow the passage of ions, water, and small proteins. For bacteria they act as an osmotic emergency release valve when the cells find themselves in a hypotonic solution, for example, through the addition of fresh water to their bathing solution. Under such circumstances water would flow into the cell to re-establish osmotic equilibrium and the cells would swell. As the cells swell the stretching of the membrane activates the opening of the MscL channels allowing ions and small proteins to diffuse out. This inhibits the further influx of water and prevents the bacterial cells from bursting. In animal cells, the osmotic equilibrium across the plasma membrane and cell volume is maintained by the Na^+,K^+-ATPase, which continually pumps Na^+ out of the cell, thus preventing water influx. However, the Na^+,K^+-ATPase isn't present in bacterial cells, hence their requirement for mechanosensitive channels.

1.4.4 Light-Gated

Light-gated channels are a recently discovered class of ion channels. At this stage, only two naturally occurring light-gated ion channels are known to exist—channelrhodopsin-1 and channelrhodopsin-2. In 2002, channelrhodopsin-1 was shown by Nagel et al. [28] to act as an H^+ channel via electrophysiological measurements after expression in *Xenopus* oocytes. In the following year, the same group showed [29] that channelrhodopsin-2 is a cation-selective ion channel. Both of these channels

contain the light-isomerizable chromophore all-*trans*-retinal, which is linked to the polypeptide chain of the protein via a protonated Schiff base. The chromophore undergoes a photoisomerization from the all-*trans* to the 13-*cis* state on absorption of a photon of the appropriate wavelength. This induces a conformational change of the protein causing the channel to open. The outward flow of ions then causes a rapid depolarization of the cell. The retinal can relax spontaneously back to the all-*trans* state within milliseconds, closing the channel and stopping the further flow of ions.

In unicellular green algae, where they occur naturally, the channelrhodopsins are involved in the control of phototaxis, that is, movement in response to light. However, their discovery has had much more far-reaching consequences than simply providing a better understanding of algal phototaxis. The expression of channelrhodopsins in other cells is proving to be a valuable tool allowing researchers to optically control the stimulation of excitable cells such as neurons. Previously, this could only be done using microelectrodes. This new field of optically controlling genetically modified cells has been given the name Optogenetics.

1.5 ION PUMPS

Whereas ion channels can be classified in terms of the gating mechanism, ion pumps can be grouped by the source of energy they utilize for ion pumping. In principle, any reaction that releases energy could be coupled to a membrane protein for the uphill pumping of ions. Here we consider only the three most widely represented sources of energy. Others do exist.

1.5.1 ATP-Activated

ATP-activated ion pumps utilize the energy released by the hydrolysis of ATP to ADP and inorganic phosphate to pump ions across membranes against their electrochemical potential gradients. The classic example is the Na^+,K^+-ATPase, which belongs to the P-type class of ion pumps. Other ion pumps belonging to this class include the Ca^{2+}-ATPase of sarcoplasmic reticulum, which plays a crucial role in muscle relaxation; the H^+,K^+-ATPase, which is responsible for the acidification of the stomach; and the H^+-ATPases in the plasma membrane of fungi and plants, which generate an H^+ electrochemical gradient across the membrane for later use as an energy source in nutrient uptake.

Depending on the relative concentrations of ATP, ADP, and inorganic phosphate, the free energy change, ΔG, for ATP hydrolysis is between -50 and $-63\,\mathrm{kJ\,mol^{-1}}$ [30]. These values indicate that it is a very favorable reaction. However, if the reaction proceeded in isolation, then this energy would be released predominantly as heat. To be utilized for the work of ion pumping, the ATPases need to possess a mechanism for the coupling of ATP hydrolysis to ion transport. The way that this occurs is by phosphoryl transfer from ATP to the protein. Thus, all the P-type ATPases possess phosphorylated intermediates as part of their reaction cycle. After ions have already bound to the protein, the intermediate which is initially produced by reaction

with ATP is a high energy state, termed E1P or E1~P. This state is able to undergo a conformational change by which it relaxes to a lower energy state, termed E2P, but in the process of this conformational change the bound ions are relocated across the membrane. Subsequently, the phosphorylated intermediate E2P releases inorganic phosphate to the surrounding aqueous medium, so that the net reaction is ATP → ADP + Pi and there is no net change in structure of the protein, but the result is that ions are pumped across the membrane.

1.5.2 Light-Activated

Light-activated ion pumps directly use energy from sunlight to pump ions across a membrane. The most prominent member of this class of ion pumps is bacteriorhodopsin, which pumps H^+ ions out of the cytoplasm of photosynthetic *Halobacteria*. Another is halorhodopsin, a light-driven chloride pump.

Bacteriorhodopsin was discovered by Walther Stoeckenius and Dieter Oesterhelt, both then working at the University of California. In 1967, Stoeckenius and Rowen [31] described the isolation of purple membrane patches from the salt-loving bacterium *Halobacterium salinarum* (formerly termed *H. halobium*), which thrives in the Californian salt lakes. A few years later, in 1971, Oesterhelt and Stoeckenius [32] showed that the purple patches contain a single protein, which they named bacteriorhodopsin because it contains the chromophore retinal, as does the visual pigment rhodopsin of humans and other animals. Furthermore, in 1973 they proposed [33] that bacteriorhodopsin acts as a light-driven H^+ pump and that the H^+ electrochemical potential gradient which the protein builds up is utilized by the cell for ATP synthesis, in agreement with Mitchell's chemiosmotic theory [23] which at the time was still controversial. This proposition was confirmed shortly afterwards by Racker and Stoeckenius [34], who reconstituted bacteriorhodopsin in vesicles together with mitochondrial ATP synthase and were able to demonstrate light-driven synthesis of ATP. These early experiments with bacteriorhodopsin were decisive in promoting the general acceptance of Mitchell's chemiosmotic theory for which he won the Nobel Prize in Chemistry only 5 years later.

The cyclic photochemical reaction that bacteriorhodopsin undergoes is very similar to that already described for the channelrhodopsins. The chromophore all-*trans* retinal is attached as a protonated Schiff base to an amino side chain of a lysine residue of the protein. On absorption of a photon the chromophore undergoes a photochemical conversion to the 13-*cis* state linked to a conformational change of the protein. The crucial point is that photon absorption causes a massive drop in the pK_a of the Schiff base, that is, a significant increase in acidity. In the dark, the pK_a of the Schiff base is around 12 and thus protonated at neutral and even slightly basic pH. After photon absorption and the conversion of retinal to the 13-*cis* state, the pK_a of the Schiff base drops by 4–5 pH units, thus promoting the loss of a proton. The drop in pK_a of the Schiff base is due to a change in its electrostatic environment caused by the protein's conformational change, that is, the proximity to other charged amino acid side chains. Subsequent to the loss of the proton, bacteriorhodopsin undergoes further conformational relaxation whereby the pK_a of the Schiff base

increases so that a proton is again taken up. After about 6 ms, bacteriorhodopsin returns to its initial conformation with retinal in its all-*trans* conformation and a protonated Schiff base.

The trick in this entire photocycle is that, because of bacteriorhodopsin's location in the membrane and the access it has to the extracellular fluid and the cytoplasm in its different conformations, the H^+ ion that is released after photon absorption is released to the extracellular fluid, whereas the H^+ that is taken up after protein conformational relaxation is taken up from the cytoplasm. Therefore, in each photocycle of bacteriorhodopsin there is a net transport of one H^+ ion out of the cell and the protein acts as a proton pump.

When H^+ ions travel back through the ATP synthase into the cell cytoplasm, the energy released is used to convert ADP to ATP. Thus, bacteriorhodopsin allows the cell to carry out a very simple form of photosynthesis, mechanistically very different from the photosynthesis of green plants, although in both energy storage in the form of an H^+ electrochemical potential gradient is used.

1.5.3 Redox-Linked

The final group of ion pumps we discuss here are those in which the energy required for ion pumping derives from an electron transfer reaction, that is, a redox reaction. The best example is probably the cytochrome-*c* oxidase, the final enzyme of the respiratory electron transport chain of mitochondria, which is a redox-linked proton pump.

The cytochrome-*c* oxidase is situated in the inner mitochondrial membrane of eukaryotic cells and in the plasma membrane of some aerobic bacteria. It catalyzes the transfer of electrons from the reduced form of cytochrome-*c* to oxygen. The overall reaction can be written as follows:

$$4\text{cyt-}c(\text{Fe}^{2+}) + 4\text{H}^+ + \text{O}_2 \rightarrow 4\text{cyt-}c(\text{Fe}^{3+}) + 2\text{H}_2\text{O}$$

Because O_2 has a higher reduction potential than cyt-c(Fe^{2+}), the reaction is spontaneous, that is, ΔG is negative. However, as described earlier for ATP hydrolysis, if this reaction were carried out in a test tube in the absence of the cytochrome-*c* oxidase, then heat would simply be generated. The cytochrome-*c* oxidase not only catalyzes the reaction, but also couples the energy released to the pumping of H^+ ions across the membrane. Therefore, rather than release energy as heat, the free energy change is used to do the work of ion pumping and store the energy in the form of an H^+ electrochemical potential gradient. When the H^+ ions then move back across the membrane through the ATP synthase and release their energy once again, ADP is converted to ATP.

In effect, the cytochrome-*c* oxidase in eukaryotic cells is performing the same function as bacteriorhodopsin in *Halobacteria*. Both generate an H^+ electrochemical potential gradient for the synthesis of ATP. They simply use different mechanisms for generating the H^+ gradient. Whereas the *Halobacteria* derive their energy for H^+ pumping directly from sunlight, eukaryotic cells derive their energy from a redox reaction.

Ultimately, the energy for the production of ATP in eukaryotic cells comes from their food intake. The oxidative phosphorylation reaction of the electron transport chain of the mitochondria utilizes electron-rich reduced nicotinamide adenine dinucleotide, NADH, produced as a result of glycolysis and the citric acid cycle. In the electron transport chain electrons are transferred in three steps from NADH to O_2, each of which involves the storage of the energy released in the form of the H^+ electrochemical gradient. The cytochrome-*c* oxidase is the enzyme which catalyzes the final step of the chain. The enzymes catalyzing the two previous steps are the NADH-coenzyme-Q reductase and the coenzyme-Q-cytochrome-*c* oxidoreductase, which catalyze the transfer of electrons from NADH to coenzyme-Q and from coenzyme-Q to cytochrome-*c*, respectively. Both of these are simultaneously proton pumps, similar to the cytochrome-*c* oxidase.

1.6 TRANSPORTERS

In principle there are two different ways in which transporters can be classified. One is according to the direction in which ions are transported. Transporters transport one ion down an electrochemical potential gradient, that is, downhill in energy, and another ion or metabolite up an electrochemical potential gradient, that is, uphill in energy. Depending on the directions of the gradients across the membrane, both transported species could move in the same direction across the membrane or they could move in opposite directions. If the directions are the same, the transporter is termed a *symporter*. If the directions are opposite, the transporter is termed an *antiporter*.

Another way of classifying transporters is according to which ion moves downhill in energy and is therefore the ion that provides the energy for the active transport of the other ion or metabolite. Different ions are used by different forms of life and in different cells or organelles, with Na^+ and H^+ ion being the most common.

1.6.1 Symporters and Antiporters

Symporters, also called cotransporters, are used by cells for the uptake of essential metabolites or nutrients into the cytoplasm. The first symporter to be identified was the Na^+/glucose cotransporter (see Section 1.1), which is present in intestinal and kidney tubule cells. Because glucose provides the cells with energy, it needs to be concentrated into the cytoplasm. The Na^+/glucose cotransporter performs this function by coupling glucose uptake to the passive diffusion of Na^+ ions through the transporter as an energy source. The high electrochemical potential gradient for Na^+ across the membrane, with high concentration in the extracellular fluid and low concentration in the cytoplasm, is created by the outward pumping of Na^+ by the Na^+,K^+-ATPase. Many other cotransporters are known for other nutrients or essential metabolites, for example, amino acids, other sugars, other organic compounds, and inorganic ions.

Antiporters, also called exchangers, are used by cells for the extrusion of ions or organic compounds from the cytoplasm into the extracellular fluid. A good example is the Na^+/Ca^{2+} exchanger, found in the plasma membrane of heart muscle cells. Evidence that such an exchange system existed was first presented by Baker et al. [35] in 1969. In muscle cells, Ca^{2+} is a signaling agent required for muscle contraction. Therefore, after each action potential the Ca^{2+} concentration in the cytoplasm must be lowered to allow muscle relaxation. By coupling Ca^{2+} extrusion from the cell to the inward flow of Na^+ ions, the Na^+/Ca^{2+} exchanger provides an effective mechanism for rapidly reducing the cytoplasmic Ca^{2+} concentration. This is, however, not the only mechanism muscle cells possess for lowering the Ca^{2+} concentration. The sarcoplasmic reticulum Ca^{2+}-ATPase also actively pumps Ca^{2+} ions into the stores of the sarcoplasmic reticulum by using the energy of ATP hydrolysis. Thus, Ca^{2+} is available for release at the next action potential and the stimulation of muscle contraction.

Another important antiporter is the Na^+/H^+ exchanger. The activity of the transporter is reversible and depends on the polarity of the electrochemical potential gradients across the membrane. In animals it utilizes the influx of Na^+ into the cytoplasm to drive the efflux of H^+ ions and thus to control the intracellular pH. In archaea, bacteria, yeast, and plants the direction of transport is the opposite such that the transporter utilizes the influx of H^+ to drive the efflux of Na^+ ions and thus to increase salt tolerance. The reason for the different directions of transport is due to the different ions that are actively pumped across the membrane in different forms of life. This is discussed in the following section.

1.6.2 Na^+-Linked and H^+-Linked

All multicellular animals possess a Na^+,K^+-ATPase in their plasma membrane. As discussed earlier (Section 1.1) this creates a Na^+ electrochemical potential gradient across the membrane which is far from equilibrium. Therefore, transporters of animal cells make use of this Na^+ gradient to provide the energy for the transport of other ions or metabolites, for example, sugars or amino acids.

Archaea, plants, fungi, and bacteria, however, generally don't possess a Na^+,K^+-ATPase. Therefore, they need other mechanisms for absorbing nutrients or extruding other ions. Usually, their transporters are coupled to an H^+ electrochemical potential gradient across the membrane. For example, the H^+ gradient built up across the plasma membrane of *H. salinarum* by the light-driven H^+-pumping of bacteriorhodopsin is used not only to drive the synthesis of ATP by the ATP synthase (described in Section 1.5.2), it is also used to drive the extrusion of Na^+ from the cytoplasm of the bacterium by a H^+-linked transporter located in the plasma membrane. In other bacteria, fungi, and plants other mechanisms for creating the H^+ gradient are employed, but it is still generally the H^+ gradient which provides the energy for nutrient uptake.

Plants and fungi possess an H^+-ATPase in their plasma membranes [36]. This is a P-type ATPase, related to the Na^+,K^+-ATPase, and which performs similar roles to the Na^+,K^+-ATPase in animals. Instead of the H^+ pumping being light-driven as it is in *Halobacteria*, the energy from ATP hydrolysis drives the pumping process. Most

of the transporters identified in plants are energized by the H^+ electrochemical gradient created by H^+-ATPases, for example, transporters for sugars, amino acids, peptides, nucleotides, and inorganic anions and cations.

Although animals don't possess H^+-ATPases in their plasma membranes, H^+ electrochemical potential gradients are produced across the inner mitochondrial membrane of every animal cell via the electron transport chain, which then drives ATP synthesis. This is one piece of evidence, amongst many others, that mitochondria evolved from ancestral bacteria (purple bacteria), that is, they were engulfed by eukaryotic cells to perform the function of ATP production. This is termed the endosymbiotic theory. In a similar fashion, the chloroplasts of green plants are thought to have evolved from ancestral cyanobacteria.

1.7 DISEASES OF ION CHANNELS, PUMPS, AND TRANSPORTERS

Diseases caused by ion channels, pumps, and transporters are widespread and as diverse as that of ion channels, pumps, and transporters themselves. Therefore, it is impossible here to provide comprehensive coverage of all the diseases with which they are associated. Instead, by selecting a small number of specific case studies, we hope to emphasize the importance of channels, pumps, and transporters for human health.

1.7.1 Channelopathies

Channelopathy is a term used in pathophysiology to describe any dysfunction of an ion channel. Many ion channel dysfunctions cause diseases in the neuromuscular system, such as epilepsy, ataxia, myotonia, and cardiac arrhythmia. Most channelopathies are inherited disorders, that is, they are the result of mutation in genes encoding channel proteins; while others are autoimmune diseases, meaning the body produces antibodies to its own channel molecules.

Cystic fibrosis was the first disorder to be discovered where the disease could be directly linked to a defect in an ion channel. The link between the channel and the disease was established by Tsui in 1989 [37]. Since then the number of disorders identified to be associated with ion channels has increased considerably. The study of ion channel diseases mostly occurs through identification of the chromosome locus of the disease and the protein coded by that gene. Once the gene has been identified, the next step is to express the mutant channel gene in, for example, HEK (human embryonic kidney cells) or *Xenopus* oocytes and study its activity using electrophysiological techniques (see Chapter 3) [37].

Table 1.1 shows that epilepsy (a disease affecting up to 1% of the world population, and which causes significant morbidity in humans) is caused by the dysfunction of several types of channels. Because of its widespread occurrence, we concentrate here on this disease [38].

Altering neuronal excitability in the brain by a mutation in an ion channel could drive a neuronal network into bursts of synchronized action potentials which

TABLE 1.1 Human Diseases Associated with Ion Channels and ABC Transporters.

Diseases Caused by Channel Disorders [38, 39]					
Channel Type	Diseases				
Voltage-gated sodium channels	Epilepsy	Cardiac disorders	Muscle disorders	Malignant hyperthermia	
Voltage-gated and inwardly rectifying potassium channels	Epilepsy	Congenital hearing loss	Episodic ataxia with myokymia syndrome	Bartter syndrome	Hyperinsulinemic hypoglycemia of infancy
Nicotinic acetylcholine receptor channels	Epilepsy	Myasthenia gravis	Congenital myasthenia	Other human central nervous system disorders	
Voltage-gated calcium channels	Hypokalemic periodic paralysis	Episodic ataxia	Familial hemiplegic migraine	Congenital stationary night blindness	Malignant hyperthermia
Diseases Caused by Transporter Disorders [40]					
Transporter Type	Diseases				
ABCB1 (MDR1)	Cancer				
ABCC1 (MRP1)	Cancer				
ABCG2 (MXR)	Cancer				
ABCB11 (SPGP)	Progressive familial intrahepatic cholestasis				
ABCB4 (MDR2)	Progressive familial intrahepatic cholestasis				

ultimately lead to an epileptic seizure; evidence of this is supported by the fact that most anticonvulsants used in clinical practice for treating epileptic seizures affect ion channels [38]. From Table 1.1 one can see three channels associated with epilepsy: voltage-gated sodium channels, brain voltage-gated potassium channels, and the neuronal nicotinic acetyl choline receptor.

In the case of the voltage-gated sodium channel, two genes, SCN1A and SCN1B, encode the α and β subunits of the channel, respectively. Mutations in both genes have been identified as possible candidates for the disorder [38, 39]. For example, generalized epilepsy with febrile seizures is caused by mutations in the SCN1A α subunit and in the SCN1B β subunit [41, 42].

Another form of epilepsy, benign neonatal epilepsy, is caused by a disorder in the function of brain voltage-gated potassium channels. In this case mutations to genes encoding two α subunits of the channels, KCNQ2 and KCNQ3, are associated with the disorder. After expression of the mutant channel in *Xenopus* oocytes, either no measurable current at all [43] or a severely reduced potassium current could be detected [44].

Nocturnal frontal lobe epilepsy is caused by a single mutation in the neuronal nicotinic acetyl choline receptor (nAChR)-α4 subunit. Expression of the mutant channel in *Xenopus* oocytes causes faster desensitization to acetyl choline and slower recovery from desensitization than that of the wild type channel [45, 46]. Mutation in the channel results in a decrease of the channel open time, a reduction of the single channel conductance, and an increase of the rate of desensitization. The possible connection between these activity changes and epilepsy is that the mutant channel might mediate the release of the inhibitory neurotransmitter GABA (γ-amino butyric acid). A reduction in nAChR function would then result in enhanced excitability of postsynaptic neurons and lower the seizure threshold [47, 48].

1.7.2 Pump Dysfunction

As an example of ion pump-related disease, we consider here disorder in the activity of the plasma membrane calcium ATPase (PMCA). The PMCA family of pumps is located in the plasma membrane and exports calcium ions from the cell; PMCA function is vital for regulating the amount of calcium within all eukaryotic cells. Improperly functioning PMCA proteins have been found to be associated with diseases, such as sensorineural deafness, diabetes, and hypertension [49].

In mammals four genes (ATP2B1–ATP2B4) encoding four proteins (PMCA1–PMCA4) have been found to be associated with some diseases. It has been proven that mutation in PMCA1, 2, and 4 is associated with disease in mice and man. For example, male mice lacking PMCA4 were found to be infertile due to loss of sperm motility. PMCA1 has a role in hypertension due to its function in the regulation of blood pressure through the alteration of calcium handling and vasoconstriction in vascular smooth muscle cells [50]. On the other hand, PMCA2 plays a role in causing ataxia and hearing loss. This was shown via the varying degrees of severity in mutants with different degrees of loss of PMCA2 function [51]. Furthermore, it was found that changes in PMCA expression are also involved in the development of other

diseases including cataract formation, carcinogenesis, diabetes, cardiac hypertension and hypertrophy, and that the severity of these diseases may be associated with subtle changes in the expression level of the PMCA isoforms in those tissues [51].

1.7.3 Transporter Dysfunction

As an example of the effect of transporter dysfunction on human health, we concentrate here on the ATP-binding cassette (ABC) transporters. ABC transporters are found in all known organisms. Approximately 1100 different transporters belonging to this family have so far been described in the literature [52]. ABC transporters typically consist of two transmembrane domains and two nucleotide-binding domains [53]. There are approximately 50 known ABC transporters in humans, defects in 14 of which can cause 13 genetic diseases (including cystic fibrosis, Stargardt disease, adrenoleukodystrophy, and Tangier disease). ABCB4 is a member of the P-glycoprotein family of multidrug resistance transporters (see Table 1.1). Six liver diseases have been found to be associated with defects in the ABCB4 gene. These are drug-induced cholestasis, adult biliary cirrhosis, phospholipid-associated cholelithiasis syndrome, progressive familial intrahepatic cholestasis type 3, transient neonatal cholestasis, and intrahepatic cholestasis of pregnancy [40].

1.8 CONCLUSION

Now that we have introduced the main categories of channels, pumps, and transporters, explained the fundamental differences in their energetics and mechanisms, and described their importance in human health, the chapters that follow are each devoted to an experimental or theoretical method for researching the mechanisms by which these important proteins function on a molecular or atomic level. The experimental methods are divided into three main groups: electrical (Chapters 2–6), spectroscopic (Chapters 7–12), radioactivity- and atomic absorption-based flux assays (Chapters 13 and 14). Finally, we conclude with a chapter on computational techniques (Chapter 15).

We begin with electrical techniques, not only because they are probably the most widely used techniques to study pump, channel, and transporter function, but also for historical reasons. The realization that muscular activity has a fundamental electrical basis goes back to the experiments of the Italian scientist Luigi Galvani [54], who in the late eighteenth century made the revolutionary discovery that contraction of the legs of frogs could be stimulated by the application of an electrical potential via metal electrodes. Although Galvani could not have known at the time that what he was actually doing was inducing the opening of voltage-gated ion channels, his experiments provided the impetus for further electrical-based experiments and he can now be considered the father of the field of electrophysiology.

Following the description of electrical methods, we turn to spectroscopic methods that are increasingly becoming more widely used methods to study pumps, channels, and transporters, sometimes in combination with electrical methods, as in the case of

voltage clamp fluorometry (discussed in Chapter 4), but also by themselves in systems for which electrical methods aren't applicable, for example, membrane fragments or small cell organelles, or in obtaining complementary mechanistic information not achievable by electrical means.

Following spectroscopic techniques, we direct our attention to the measurement of fluxes of ions and substrates across the cell plasma membranes based on the detection of radioactivity (Chapter 13) or atomic absorption (Chapter 14). No book on ion transport function would be complete without a discussion of the use of radioactivity. As described in Section 1.1, the use of radioisotopes, which became possible in the late 1930s, enabled rapid major advances in the ion transport field due to the far greater accuracy that could be achieved. In particular, radioisotopes allowed the hypothesis of Na^+ impermeability of cell membranes to be finally buried, a hypothesis which had been hampering progress for decades. As will be described in Chapters 13 and 14, radioactivity-based and atomic absorption-based techniques are still extremely powerful research tools and are particularly useful in the study of charge-neutral transport, when ion transport generates no electrical potential difference across the membrane. Finally, Chapter 15 describes the application of theoretical methods, which have only recently become feasible due to major advances in computer hardware and simulation procedures together with the increasing availability of high-resolution crystal structures of membrane proteins.

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