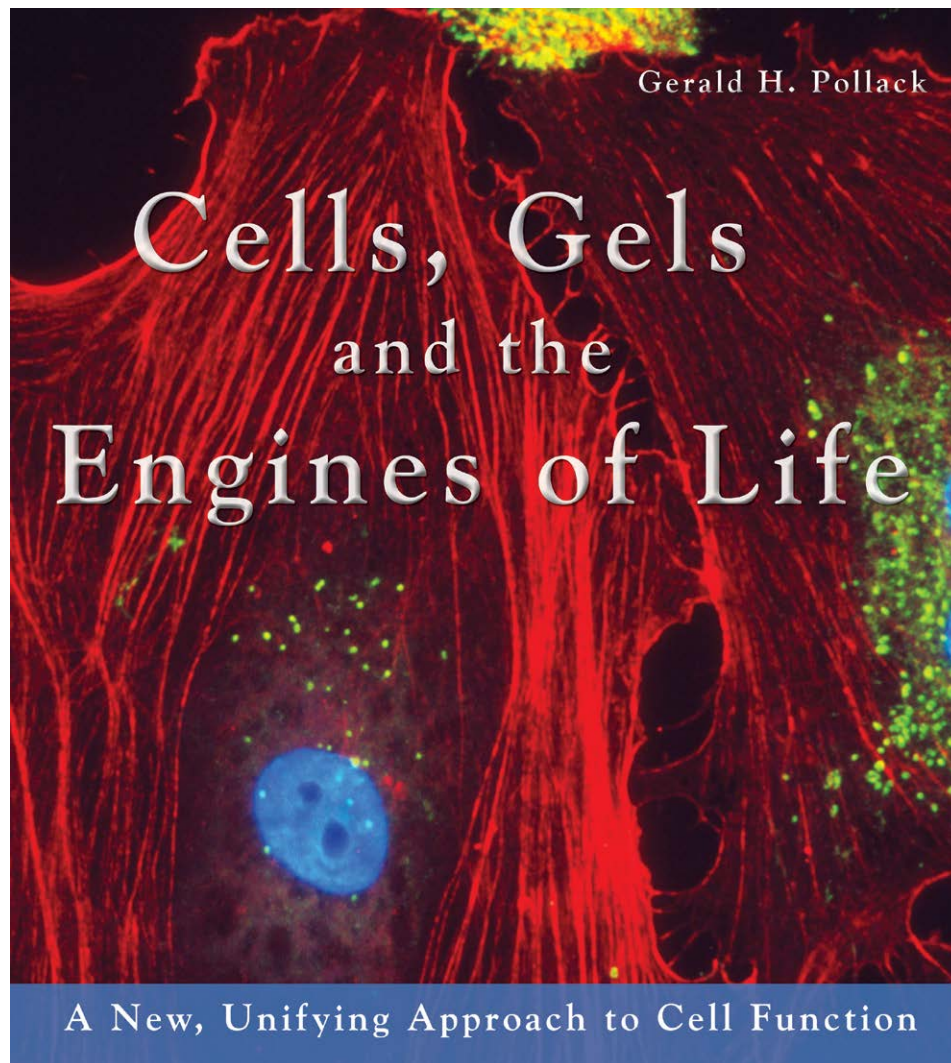




Cells, Gels and the Engines of Life by **Gerald H. Pollack**

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Published by Ebner & Sons Publishers

ISBN Hardcover: 0-9626895-1-3

ISBN Paperback: 0-9626895-2-1

SAMPLE READER consisting of Chapters 1, 2, and Index

DEBUNKING MYTHS

Long ago, scientists believed that the center of the universe was the earth: The sun could be seen to traverse the heavens, so it was logical to conclude that the earth must lie at the center point.

But this view eventually encountered difficulties. As the growth of mathematics increased the power of astronomy, it became possible to compute orbital pathways. The planets' paths around the earth turned out to be less simple than anticipated; each planet followed an orbit called an epicycle, which was sufficiently intricate to imply that something was surely amiss.

What was amiss is no longer a mystery. Although the persistent notion of an earth-centered universe may gratify our collective egos, Galileo showed that it was the sun that held this honor. With the sun at the solar system's center, orbital paths no longer required complex epicycles; they became a lot simpler. What had earlier seemed a reasonable hypothesis supported by seemingly indisputable visual observation, turned out to be dead wrong. A complicated paradigm was replaced by a simpler one.

IS LIFE REALLY ANY DIFFERENT NOW?

In the field of cell biology at least, complicated paradigms raise similar concern. On the surface everything seems to be in order. Virtually all known cellular processes are by now accounted for by well-described mechanisms: ions flow through channels; solutes are transported by pumps; vesicles are

moved by motors; *etc.* For every problem there is a solution. But as we shall see as we probe beneath the surface of these solutions, a bewildering level of complexity hints at a situation that could parallel the epicycles.

I propose to step back and regroup. For genuine progress, foundational concepts must be unquestionably sound; otherwise an edifice of understanding may rise over a crevasse of uncertainty—no apparent problem until the edifice grows weighty enough to crack the foundation and tumble into the abyss. Firm ground needs to be identified.

I begin by considering two elements thought to be fundamental to cell function: membrane pumps and channels. Pumps transport solutes across the cell boundary against their respective concentration gradients. Channels permit the solutes to trickle back in the opposite direction. Through a balance between pump-based transport and channel-based leakage, the characteristic partitioning of solutes and ions is thought to be established.

Table 1.1. Concentration of principal ions inside and outside of a typical mammalian cell			
Ion	Molecular Weight	Intracellular Concentration (mM)	Extracellular Concentration (mM)
Na	w	5-15	145
K	39	140	5
Cl	35.5	5-15	110

Thus, potassium concentration is relatively higher inside the cell, and sodium is relatively higher outside (Table 1.1).

That pumps and channels exist seems beyond doubt—or to put it more precisely, the existence of proteins with pump-like or channel-like features cannot be doubted. Genes coding for these proteins have been cloned, and the proteins themselves have been exhaustively studied. There can be no reason why their existence might be challenged.

Where some question could remain is in the functional role of these proteins. What I will be considering in this chapter is whether these proteins really mediate ion partitioning. Because a “pump” protein inserted into an artificial membrane can translocate an ion from one side of the membrane to the other, can we be certain that ion partitioning in the living cell necessarily occurs by pumping?

This task of checking this presumption may in this case be approached through the portal of historical perspective. Scientists on the frontier often dismiss history as irrelevant but in this particular instance a brief look into the trail of discovery is especially revealing.

ORIGINS

The emergence of pumps and channels was preceded by the concept of the cell membrane. The latter arose during the era of light microscopy, prior to the time any such membrane could actually be visualized. Biologists of the early nineteenth century observed that a lump of cytoplasm, described as a “pulpy, homogeneous, gelatinous substance” (Dujardin, 1835) did not mix with the surrounding solution.

To explain why this gelatinous substance did not dissolve, the idea arose that it must be enveloped by a water-impermeant film. This film could prevent the surrounding solution from permeating into the cytoplasm and dissolving it. The nature of the membranous film had two suggested

variants. Kühne (1864) envisioned it as a layer of coagulated protein, while Schültze (1863) imagined it as a layer of condensed cytoplasm. Given the experimental limitations of the era, the nature of the putative film, still not visualizable, remained uncertain.

The idea of an invisible film was nevertheless attractive to many of the era's scientists, and was increasingly conferred with special attributes. Thus, Theodore Schwann (1839) viewed this film as "prior in importance to its contents." The membrane grew in significance to become the presumed seat of much of the cell's activity. Yet this view was not accepted by all. Max Schültze, often referred to as the father of modern biology, discounted the evidence for a cytoplasmic film altogether, and instead regarded cells as "membraneless little lumps of protoplasm with a nucleus" (Schültze, 1861). In spite of Schültze's prominence, the concept of an enveloping membrane held firm.

The modern idea that the membrane barrier might be semi-permeable came from the plant physiologist Wilhelm Pfeffer. Pfeffer was aware of the ongoing work of Thomas Graham (1861) who had been studying colloids, which are large molecules suspended indefinitely in a liquid medium—*e.g.*, milk. According to Graham's observations, colloids could not pass through dialysis membranes although water could. To Pfeffer, colloids seemed to resemble the cytoplasm. If the dialysis membrane were like the cell membrane, Pfeffer reasoned, the cell interior would not dissipate into the surrounding fluid even though the membrane might still be water-permeable. Thus arose the idea of the semi-permeable membrane.

Pfeffer took up the semi-permeable membrane idea and pursued it. He carried out experiments on membrane models made of copper ferrocyanide, which acted much like dialysis membranes in that they could pass water easily but solutes with great difficulty. It was on these experiments that Pfeffer based the modern cell-membrane theory (Pfeffer, 1877). The membrane at this stage was presumed permeable to water, but little else.

Although Pfeffer's theory held for some time, it suffered serious setbacks when substances presumed unable to cross the membrane turned out to cross. The first and perhaps most significant of these substances was potassium. The recognition, in the early twentieth century, that potassium could flow into and out of the cell (Mond and Amson, 1928; Fenn and Cobb, 1934), prompted a fundamental rethinking of the theory.

ORIGIN OF THE CHANNEL

Faced with the need to explain the potassium-permeability issue, Boyle and Conway (1941) proposed an elegant solution: the potassium channel. Since the hydrated potassium ion was known to be smaller than the hydrated sodium ion, 3.8 Å vs. 5 Å, Boyle and Conway proposed trans-membrane channels of critical size—large enough to pass potassium and its shell of associated water, but small enough to exclude sodium with its shell. The membrane was effectively a sieve that passed small ions, but excluded larger ones.

The Boyle-Conway atomic sieve theory was attractive in that it could also account for several known features of cell behavior without too much difficulty. It explained the accumulation of potassium inside the cell as an attraction to the cell's negatively charged proteins (a so-called Donnan effect). It explained the cell potential as arising from a charge separation across the membrane (a capacitive effect). And it accounted for the changes of cell volume that could be induced by changes of external potassium concentration (an osmotic effect). The sieve theory seemed to explain so much in a coherent manner that it was immediately granted an exalted status.

But another problem cropped up, perhaps even more serious than the first. The membrane turned out to be permeable also to sodium (Fig. 1.1). The advent of radioactive sodium made it possible to trace the path

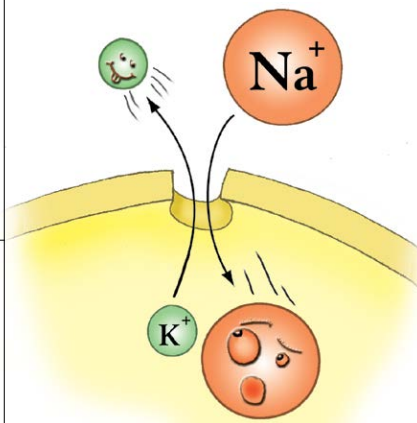


Figure 1.1. Atomic sieve theory. The size of the channel was postulated to be critical for passing potassium and blocking sodium. Observed passage of sodium compromised the theory.

of sodium ions, and a cadre of investigators promptly found that sodium did in fact cross the cell boundary (Cohn and Cohn, 1939; Heppel, 1939, 1940; Brooks, 1940; Steinbach, 1940). This finding created a problem because the hydrated sodium ion was larger than the channels postulated to accommodate potassium; sodium ions should have been excluded, but they were not. Thus, the atomic-sieve theory collapsed.

Collapse notwithstanding, the transmembrane-channel concept remained appealing. One had to begin somewhere. A channel-based framework could circumvent the sodium problem with separate channels for sodium and potassium: if selectivity were based on some criterion other than size, then distinct channels could suffice. A separate channel for sodium could then account for the observed leakage of sodium ions into the cell.

But leakage of sodium introduced yet another dilemma, of a different nature. Sodium could now pass through the channel, flowing down its concentration gradient and accumulating inside the cell. How then could intracellular sodium remain as low as it is?

ORIGIN OF THE PUMP

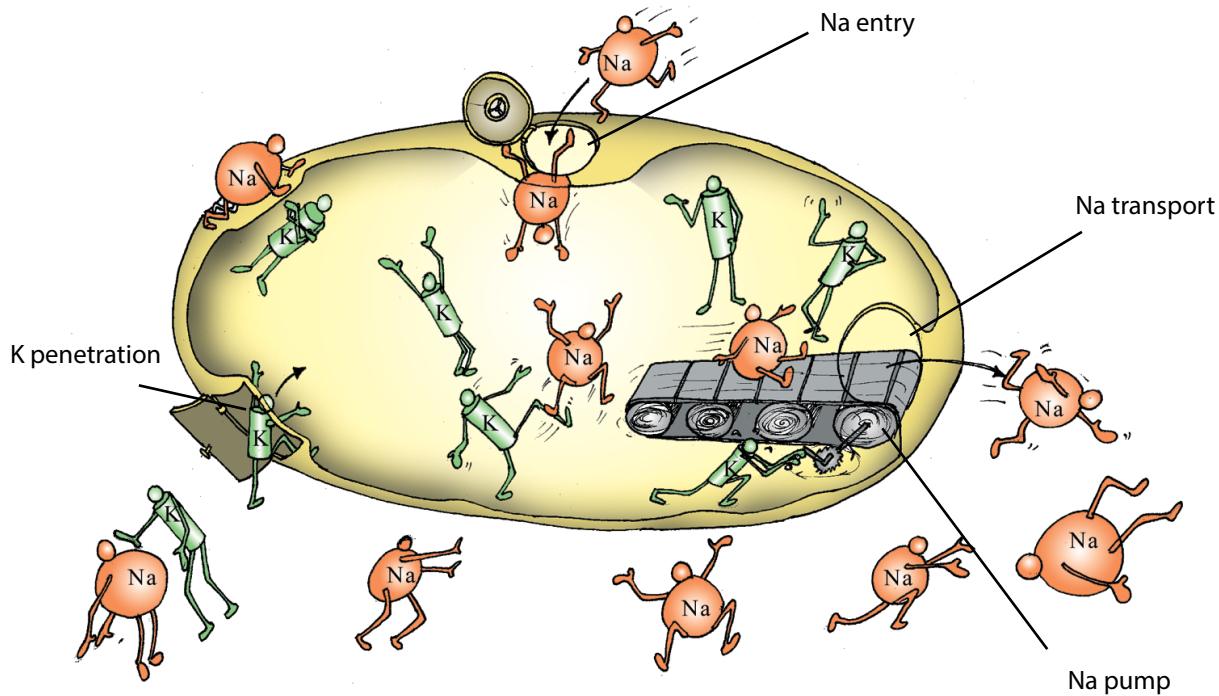
The solution was to pump it out. In more-or-less the same manner as a sump-pump removes water that has leaked into your basement, a membrane pump was postulated to rid the cell of the sodium that would otherwise have accumulated inside.

The idea of a membrane pump actually originated before the sodium problem. It began at the turn of the last century with Overton, a prominent physiologist who had advanced the idea that the membrane was made of lipid. Realizing that some solutes could cross an otherwise impermeable lipid membrane, Overton postulated a kind of secretory activity to handle these solutes. Through metabolic energy, the membrane could thus secrete, or pump, certain solutes into or out of the cell.

The pump concept resurfaced some forty years later (Dean, 1941), to respond specifically to the sodium-permeability problem. Dean did not have a particular pumping mechanism in mind; in fact, the sodium-pump was put forth as the least objectionable of alternatives. Thus, Dean remarked, “It is safer to assume that there is a pump of unknown mechanism which is doing work at a constant rate excreting sodium as fast as it diffuses into the cell.” With this, the sodium pump (later, the Na/K exchange pump) came decidedly into existence.

By the mid-twentieth century, then, the cell had acquired both channels and pumps. With channels for potassium and sodium, along with pumps to restore ion gradients lost through leakage, the cell’s electrophysiology seemed firmly grounded. Figure 1.2 says it all.

Figure 1.2. The sodium pump, adapted from a drawing by Wallace Fenn (1953), one of the field’s pioneers.



REFLECTIONS

Why have I dragged you through so lengthy a review? My purpose was to demonstrate how channels and pumps arose. They came into being not because some alert scientist stumbled upon them during a groundbreaking session at the electron microscope, but as *ad hoc* hypotheses needed to patch otherwise flagging theories. The channel arose when a putatively ion-impermeant membrane was found to pass potassium; the channel could pass potassium while properly excluding sodium and other larger hydrated ions. Then, sodium was found to enter the cell and instead of reconsidering the channel concept, a second channel specific for sodium was postulated. Sodium permeability also implied a persistent leak into the cell. To keep gradients from collapsing, a sodium pump was postulated.

Once this hypothetical framework gained a foothold, it expanded boundlessly. For the same reason that a sodium pump was needed, it became evident that pumps for other solutes were needed as well. Virtually all of the cell's solutes partition far out of electrochemical equilibrium (Stein, 1990) and therefore need to be pumped. Hydrogen-ion pumps, calcium pumps, chloride pumps, and bicarbonate pumps to name a few, soon came into being over and above the postulated sodium/potassium pumps. Yet, even at the height of this intense activity, Glynn and Karlish (1975) in their classic review had to reluctantly admit that notwithstanding an enormous thrust of experimental work on the subject, still no hypothesis existed to explain how pumps pump.

The channel field exploded similarly. With the advent of the patch-clamp technique (see below) in the late 1970s, investigators had gained the capacity to study what appeared to be single ion channels. It seemed for a time that new channels were being identified practically monthly, many of them apparently selective for a particular ion or solute. The number of channels has risen to well over 100. Even water channels have come into being (Dempster *et al.*, 1992). Elegant work was carried out to try to understand how channels could achieve their vaunted selectivity (Hille,

1984). At least some channels, it appeared, could pass one ion or solute selectively, while excluding most others.

Given the astonishing expansion of activity in these fields, could there be any conceivable basis for doubt? Mustn't the concepts of pumping and channeling be as firmly grounded as any biological principle?

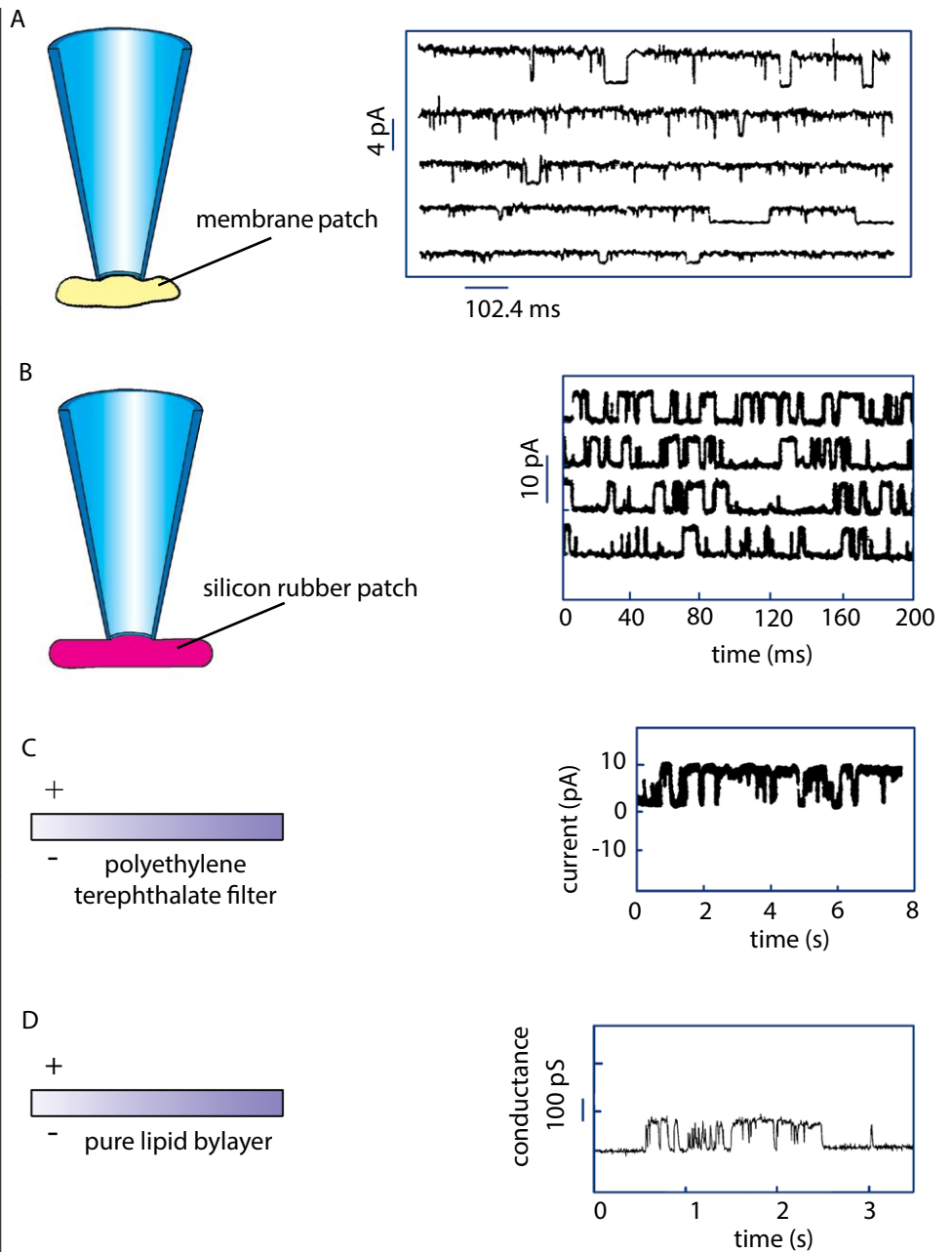
It is tempting to answer by placing side-by-side the key experiments originally adduced to confirm pumping and channeling together with the published challenges of those experiments (Troshin, 1966; Hazlewood, 1979; Ling, 1984, 1992). I hesitate to recapitulate that debate because the challenges are largely technical. Readers willing to invest time in acquiring familiarity with technical details are invited to consult these sources and consider whether the published concerns are valid or not.

Another approach is to consider evidence that could potentially lie in conflict with these concepts. Unlike mathematical theorems, scientific theories cannot be proven. No matter how much evidence can be marshaled in support of a theory, it is always possible that some new piece of evidence will be uncovered that does not fit, and if such evidence is both sound and fundamental, the theory may require reconsideration. As we shall see in the next sections, certain basic questions concerning pumps and channels have not yet been adequately dealt with.

CHANNELS REVISITED

The existence of single ion channels appeared to be confirmed by ground-breaking experiments using the patch-clamp technique. In this technique the tip of a micropipette is positioned on the cell surface. Through suction, a patch of membrane is plucked from the cell and remains stuck onto the micropipette orifice (Fig. 1.3A). A steady bias voltage is placed across the patch, and the resulting current flow through the patch is measured. This current is not continuous; it occurs as a train of discrete pulses. Because the pulses appear to be quantal in size, each pulse is assumed to

Figure 1.3. "Single channel" currents recorded in situations depicted at left of each panel: (A) after Tabcharani et al. (1989); (B) after Sachs and Qin (1993); (C) after Lev et al. (1993); (D) after Woodbury (1989). Note the similarity of experimental records, implying that the discrete currents are not necessarily related to features specific to biological channels.



correspond to the opening of a single ion channel.

This dazzling result has so revolutionized the field of membrane electrophysiology that the originators of the technique, Erwin Neher and Bert Sakmann, were awarded the Nobel Prize. The observation of discrete events would seem to confirm beyond doubt that the ions flow through discrete channels.

Results from the laboratory of Fred Sachs, on the other hand, make one wonder. Sachs found that when the patch of membrane was replaced by a patch of silicon rubber, the discrete currents did not disappear (Sachs and Qin, 1993); they remained essentially indistinguishable from those measured when the membrane was present (Fig. 1.3B). Even more surprisingly, the silicon rubber sample showed ion-selectivity features essentially the same as the putative membrane channel.

A similarly troubling observation was made on polymer samples (Lev *et al.*, 1993). Current flow through synthetic polymer filters was found to be discrete, just as in silicon rubber (Fig. 1.3C). The filters also showed features commonly ascribed to biological channels such as ion selectivity, reversal potential, and gating. Yet, the sample was devoid of any protein or lipid.

In yet another set of experiments, channel-like behavior was observed in pure lipid-bilayer membranes (Woodbury, 1989). Following brief exposure to large concentrations of lipid vesicles ejected from a pipette tip approximately 0.5 mm distant, these membranes showed typical channel-like fluctuations (Fig. 1.3D). Conductance changed in ways usually considered to be indicative of reconstituted protein channels—including step conductance changes, flickering, ion selectivity, and inactivation. But no channels were present; the membranes contained only lipid.

What are we to do with such observations? It is clear from these three studies that the discrete currents previously taken to confirm the existence

of single biological channels seem to be general features of current flow through small samples. The currents presumably arise from some common feature of these specimens that is yet to be determined, but evidently not from single channels since they are absent. The channels may exist—but the prime evidence on which their existence is based is less than conclusive.

Ironically, the silicon-rubber test had actually been carried out as a control in the original patch clamp studies (Neher *et al.*, 1978). The authors did sometimes note “behavior contrary” to what was expected (p. 223); but such behavior was dismissed as having arisen from irregularities of the pipette tip. The possibility that small samples in general might give rise to channel-like behavior was apparently not considered.

Setting aside the above-mentioned concern, a second point to consider is the manner in which the channel achieves its specificity. Channels exist for each one of the cell’s ions; additional channels exist for amino acids, peptides, toxins, and sugars, most of these being otherwise unable to cross the lipid bilayer; and as I mentioned, there are also channels for water. Thus, a plethora of channels exists, most engineered to be solute-specific. How is such specificity achieved?

To explain such exquisite specificity, models of some complexity have evolved (Hille, 1984; 1992). One model contains 16 different transition states, plus additional sub-states. Another contains 27 states. Most models are sufficiently complex that the solution requires numerical methods. Indeed, calculating the trajectory of a molecule diffusing through a channel during a 100-picosecond time window is the work of a supercomputer. Thus, models of daunting complexity are required for understanding the channels’ apparent selectivity. It is not a simple process.

The naive question nevertheless lingers: How is it that small solutes do not pass through large channels? To imagine how one or two small ions might be excluded from a large channel as a result of a distinctive electric field distribution is not too difficult to envision. But the theory implies

that smaller solutes should be excluded as a class—otherwise, independent channels for these solutes would not be required. This enigma harks of the dog-door analogy (Fig. 1.4). Why bother adding a cat door, a ferret door, a hamster door and a gerbil door when these smaller animals could slip readily through the dog door? Must some kind of repellent be added for each smaller species?

The seriousness of the size problem is illustrated by considering the hydrogen ion. The hydrogen ion is only about 0.5 Å in diameter (the hydrated ion somewhat larger). The sodium-channel orifice is at least 3 - 5 Å across, and channels specific for some of the larger solutes must have



Figure 1.4. Dog-door analogy. Door is large enough to pass all smaller species.

a minimum orifice on the order of 10 - 20 Å. How is it possible that a 20 Å channel could exclude a diminutive hydrogen ion? It is as though a two-foot sewer pipe that easily passes a beach ball could at the same time exclude golf balls, as well as tennis balls, billiard balls, *etc.*

Arguably, the situation is not so black and white. Textbook depictions of the channel as a hollow tube oversimplify the contemporary view of the channel as a convoluted pathway; and the process of selectivity is thought to rest not on size *per se* but on some complex interaction between the solute's electric field and structural features of the channel's filter (*e.g.*, Doyle *et al.*, 1998). Also, channel selectivity is not absolute (Hille, 1972). Nevertheless, the issue of passing only one or a few among a field of numerous possible solutes including many smaller ones remains to be dealt with in a systematic manner. And the issue of non-biological samples producing single-channel currents certainly needs to be evaluated as well. What could all this imply?

PUMPS REVISITED

Like channels, pumps come in many varieties and most are solute-specific. The number easily exceeds 50. The need for multiple pumps has already been dealt with: unless partitioning between the inside and outside of the cell is in electrochemical equilibrium, pumping is required. Because so few solutes are in equilibrium, one or more pumps are necessary for each solute.

A question that arises is how the cell might pump a solute it has never seen. Antibiotics, for example, remain in high concentration outside the bacterial cell but in low concentration inside. Maintaining the low intracellular concentration implies the need for a pump, and in fact, a tetracycline pump for *E. coli* has been formally proposed (Hutchings, 1969). A similar situation applies for curare, the exotic arrow poison used experimentally by biophysicists. Because curare partitioning in the

muscle cell does not conform to the Donnan equilibrium, a curare pump has been proposed (Ehrenpries, 1967). To cope with substances it has never seen, the cell appears to require pumps over and above those used on a regular basis—on reserve.

How is this possible? One option is for existing pumps to adapt themselves to these new substances. But this seems illogical, for if they could adapt so easily why would they have been selective to begin with? An alternative is for the cell to synthesize a new pump each time it encounters a foreign substance. But this option faces the problem of limited space: Like the university parking lot, the membrane has just so many spaces available for new pumps (and channels). Given chemists' ability to synthesize an endless variety of substances—10 million new chemical substances have been added to the American Chemical Society's list of molecules during the last quarter century alone (*N. Y. Times*, Feb 22, 2000)—how could a membrane already crowded with pumps and channels accommodate all that might eventually be required? Could a membrane of finite dimension accommodate a potentially infinite number of pumps?

A second question is how the cell musters the energy required to power all of its pumps. Where might all the ATP come from? Since ions and other solutes cross the membrane continually even in the resting state (in theory because of sporadic channel openings), pumps must run continuously to counteract these leaks. Pumping does not come free. The sodium pump alone has been estimated, on the basis of oxygen-consumption measurements, to consume 45 - 50% of all the cell's energy supply (Whittam, 1961). Current textbooks estimate a range of 30 - 35%.

To test whether sufficient energy is available to power pumping, a well-known experiment was carried out long ago by Ling (1962). Ling focussed on the sodium pump. The idea was to expose the cell to a cocktail of metabolic poisons including iodoacetate and cyanide, and to deprive it of oxygen—all of which would deplete the cell of its energy supply and effectively pull the pump's plug. If these pumps had been responsible for

maintaining sodium and potassium gradients, the gradients should soon have collapsed. But they did not. After some eight hours of poison exposure and oxygen deprivation, little or no change in cellular potassium or sodium was measurable.

Ling went on to quantitate the problem. He computed the residual energy—the maximum that could conceivably have been available to the cell following poisoning. This residual was compared to the energy required to sustain the ion gradient, the latter calculable from the known sodium-leak rate. Using the most generous of assumptions, a conservative estimate gave an energy shortfall of 15 to 30 times (Ling, 1962). The pump energy needed to sustain the observed gradient, in other words, was concluded to be at least 15 to 30 times larger than the available energy supply.

This conclusion stirred a good deal of debate. The debate was highlighted in a *Science* piece written by the now well-known science writer Gina Kolata (1976), a seemingly balanced treatment that gave credence to the arguments on both sides. Kolata cited the work of Jeffrey Freedman and Christopher Miller who had challenged Ling's conclusion about the magnitude of the energy shortfall. Ling's late-coming rebuttal (1997) is a compelling "must-read" that considers not only this specific issue but also the process of science. The energy-shortfall claim was nevertheless left to gather dust with the advent of pump-protein isolation—forgotten by all but a modest cadre of researchers who have remained steadfastly impressed by the arguments (*cf.* Tigyi *et al.*, 1991).

In retrospect, any such niggling debate about the magnitude of the sodium-pump-energy shortfall seems academic, for it is now known that numerous other pumps also require power. Over and above sodium and potassium, the cell membrane contains pumps for calcium, chloride, magnesium, hydrogen, bicarbonate, as well as for amino acids, sugars, and other solutes. Still more pumps are contained in organelle membranes inside the cell: In order to sustain intra-organelle ion partitioning, organelles such as the mitochondrion and endoplasmic reticulum contain pumps similar

to those contained in the surface membrane. Given leak rates that are characteristically proportional to surface area, we are not speaking here of trivial numbers of pumps: Liver cell mitochondria contain 20 times the surface area of the liver cell membrane (Lehninger, 1964), and the area of the muscle's sarcoplasmic reticulum is roughly 50 times that of the muscle cell membrane (Peachey, 1965). Membranes of such organelles must therefore contain pumps in numbers far higher than those of the cell membrane—all requiring energy.

In sum, pumping faces obstacles of space and energy. The membrane's size is fixed but the number of pumps will inevitably continue to grow. At some stage the demand for space could exceed the supply, and what then? Pumping also requires energy. The Na/K pump alone is estimated to consume an appreciable fraction of the cell's energy supply, and that pump is one of very many, including those in internal membranes. How is the cell to cope with the associated energy requirement?

COULD CHANNEL AND PUMP PROTEINS PLAY ANOTHER ROLE?

The sections above have outlined certain obstacles faced by the pump – channel paradigm. But proteins exhibiting pump-like or channel-like behavior have been isolated and their existence needs to be explained. If not specifically for pumping and channeling, why might they be present?

One plausible hypothesis is that they exist for some different purpose. Given their superficial location, “pump” and “channel” proteins could trigger a chain of events leading ultimately to action in an intracellular target. Conformational changes are known to occur not only in pump and receptor proteins but in channel proteins as well (Kolberg, 1994). If such changes were to propagate inward, the pump or channel protein would effectively play the role of a receptor.

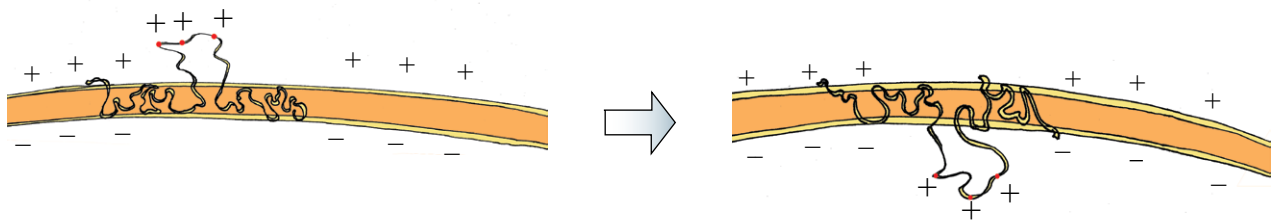
A scenario of this sort need not contradict the proteins' classical “pump-

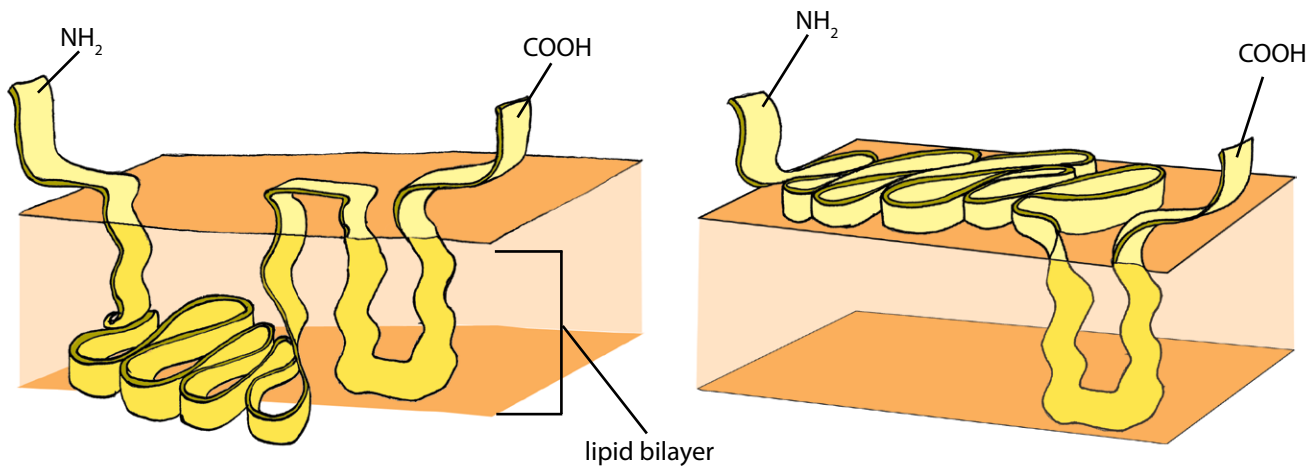
Figure 1.5. Translocation of charges across the membrane will be registered as current pulses.

ing” or “channeling” action: As the conformational change proceeds, any bound ion will shift in space along with the protein (Fig. 1.5). If the charge shifts against the voltage gradient, the result will be interpreted as pumping; if it shifts with the gradient the ion will be presumed to have passed through a channel. Thus, pumping or channeling would be a natural inference, even though the protein’s functional role is as neither a pump nor a channel.

A good example to illustrate this kind of behavior is colicin Ia, a toxin molecule that insinuates into bacterial membranes. As it does, it is thought to create a channel that allows ions to pass, thereby collapsing ion gradients and killing the cell. The protein’s action is associated with substantial conformational change (Fig. 1.6); some 50 amino acids flip from one side of the membrane to the other—along with their bound ions. Such charge shift would constitute a pulse of current similar to the currents depicted in Figure 1.3; hence, channeling is implicit. Whether such “channel” current mediates the protein’s toxic function, however, is less certain. Toxicity could as well lie in the protein’s altered configuration, inhibiting some vital process through interaction with cell proteins.

Another example is rhodopsin. Rhodopsin is a retinal receptor molecule that undergoes conformational change in order to signal the presence of light. Rhodopsin exists in another form called bacteriorhodopsin. Also driven by absorbed light energy, bacteriorhodopsin can translocate protons across the bacterial cell membrane. Thus, rhodopsin is a light-driven receptor while bacteriorhodopsin is presumed to be a light-driven pump. Again, the charge movement observed in bacteriorhodopsin may not





necessarily be the main event—the protein could function as a receptor of light just as rhodopsin does in the retina, triggering a response through conformational change (Lewis *et al.*, 1996). As with the channeling action of colicin Ia, the pumping action of bacteriorhodopsin might then be an incidental byproduct, and not necessarily the primary event.

Whether the observed pumping or channeling seen in such molecules could be of functional significance would depend on their magnitude: If the “pump” molecule translocates relatively few ions, its contribution to cellular ion separation would be small. And if the “channel” fails to carry the lion’s share of ion traffic through the membrane, it too would play little or no role in partitioning. Even though experimentally observable, pumping and channeling by these proteins would then remain functionally insignificant—much like the heat generated by a light-bulb.

It seems, then, that this section’s conundrum may be resolvable. Although tests imply that the proteins under consideration can “pump” or “channel,” such processes may be incidental to the proteins’ main functional role as receptors. Receptor proteins are often closely linked to pump and channel proteins in order to “modulate” their activity; here they merge into a single unit whose contributions to ion partitioning may be entirely secondary.

Figure 1.6. Channel protein that opens by flipping from one side of the membrane to the other. After Slatin *et al.* (1994).

CONCLUSION

Some bold leaps have been taken in this chapter. We began by granting ourselves license to explore two basic elements of modern cell biology—channels and pumps. As we reviewed their origin, we found that they arose as postulates, put forth to rescue attractive theories that would otherwise have collapsed.

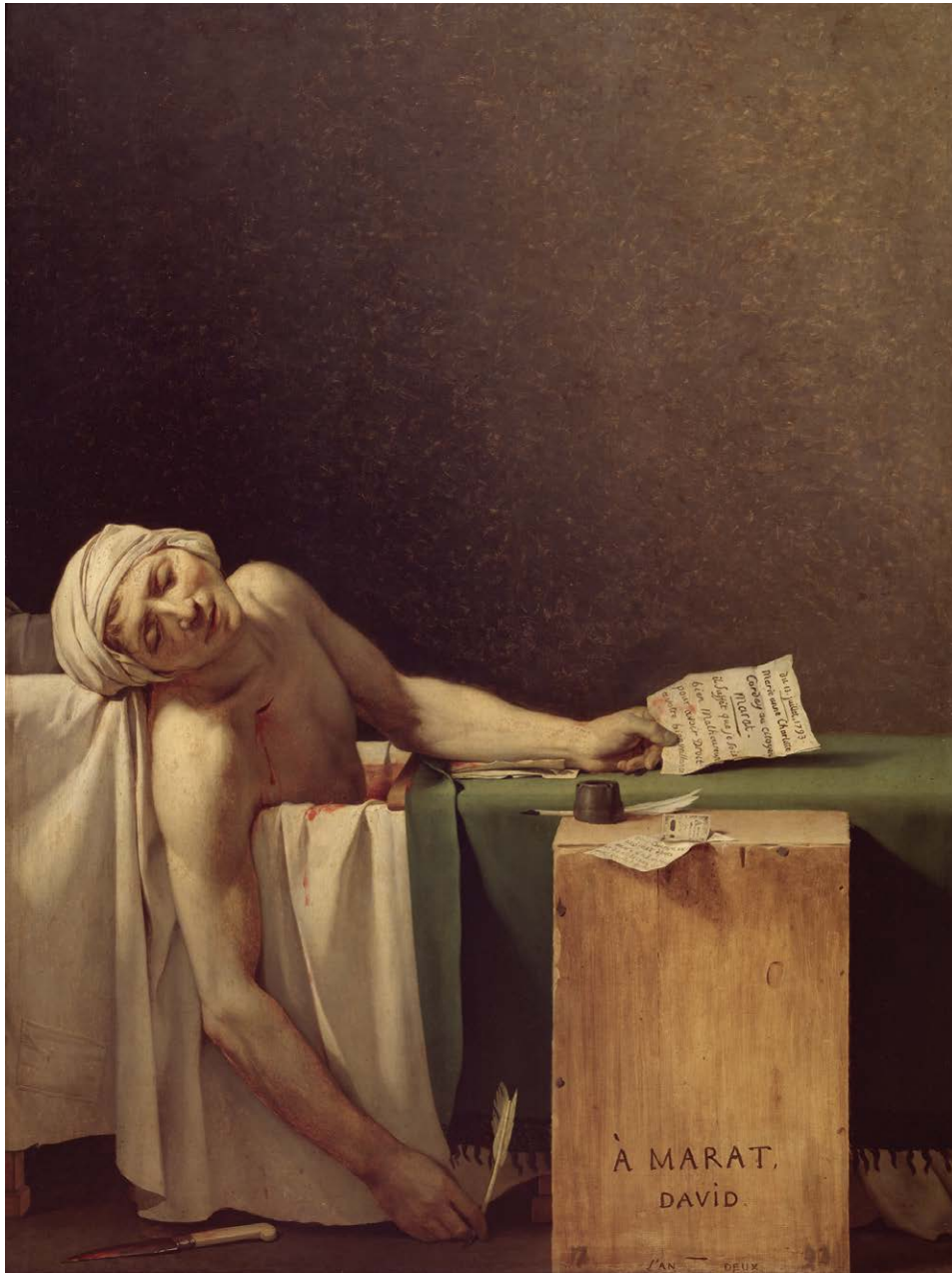
The framework surrounding those postulates then grew in complexity. Channels and pumps multiplied rampantly in number and their features grew devilishly intricate. In order to achieve selectivity, the channel needed many states and sub-states; and the pump was required to handle substances it had never before seen. These complexities hinted that something could well be amiss.

Some things were indeed amiss, or at least questionable. For the channels it was the lead provided by the patch-clamp experiments. Those experiments had been taken as proof of the existence of discrete biological channels, but that evidence has been thrown into doubt by the demonstration that similar results could be obtained when channels were absent. Also considered was the selectivity issue. It was not clear how the channel could pass one solute primarily, while systematically excluding others of the set—particularly its smaller members (the dog-door problem).

Questions were also raised about pumps. One issue is the means by which a membrane of finite surface area could accommodate a continually growing number of pumps (and channels)—what happens when space runs out? A second issue is the nettlesome one of energy-balance: if the cell's energy supply is marginally adequate to handle sodium pumping, what resources are available to power all of the rest of the many pumps?

Although this chapter's goal was to begin constructing a functional edifice, the challenge of finding solid foundational ground has not yet been

met. The foundation remains uncertain. Nor can the soundness of sub-structural layers be presumed, for the pump and channel questions seem profound enough to hint that the problems could originate more deeply.



The Death of Marat, 1793, by Jacques Louis David.

2

CHAPTER 2

THE CROAK OF THE DYING CELL

Given the turn of events in Chapter 1, we need to ask whether the uncertainties end there. Pumps and channels did not emerge in a vacuum. They arose out of an established conceptual framework and it may be that the original sin lies within the framework—much like the epicycles.

The framework in question is the cell membrane. Pumps came into being when a membrane that was presumed impermeant to solutes was found to be permeant. At first it was a single solute, then another, and then additional ones. Instead of abandoning the notion of impermeance, the passage of each solute was accommodated by presupposing another channel (and pump). Serious consideration was not given to the alternative possibility—that the continuous barrier framework itself might be erroneous.

When dealing with membranes, I recognize we are treading on hallowed ground. The continuous phospholipid-barrier concept has become so deeply ingrained in modern thinking that merely putting it on the table for discussion seems akin to reconsidering the virtues of motherhood. Yet, as a major limb of the logic tree, the issue cannot be ignored.

To approach this issue, we consider what happens when the membrane is disrupted. If ion partitioning (Table 1.1) requires a continuous barrier, violating the barrier should collapse these critical gradients. The violated cell should also lose enzymes and fuel, metabolic processes should grind to a halt, and the cell should be brought quickly to the edge of death. On the other hand, if the barrier were not continuous to begin with then

membrane disruption could prove relatively inconsequential.

WANTON ACTS

To disrupt the membrane experimentally, scientists have developed means uncannily similar to those developed to inflict wounds on humans, namely cellular swords and guns. Such instruments are not concocted by enraged scientists bent on revenge; they are designed to probe. Consider the three implements shown in Figure 2.1:

- The *microelectrode* (or micropipette) is a tapered glass cylinder filled with an electrolyte solution. The tip is plunged into the cell in order to probe its electrical properties. The microelectrode tip may seem diminutive by conventional standards, but to the 10- μm cell, invasion by a 1- μm probe is not entirely dissimilar to a human torso being impaled by a fence post.

- *Electroporation* is a widely used method of punching multiple holes in the cell membrane. The holes are created by shotgunning the cell with a barrage of high-voltage pulses, leaving the membrane riddled with orifices large enough to pass genes, proteins and other macromolecules.

- The *patch-clamp* method involves the plucking of a 1- μm patch of membrane from the cell for electrophysiological investigation.

Although these insults may cause fatal injuries in some cells, the surprise, as we see below, is that they are not consistently consequential.

Consider the microelectrode plunge. Not only does impalement violate the membrane, but it also mangles organelles unfortunate enough to lie along the track of penetration. The anticipated surge of ions, proteins, and metabolites might be thwarted if the hole were kept plugged by the microelectrode shank, but this cannot always be the case. For example,

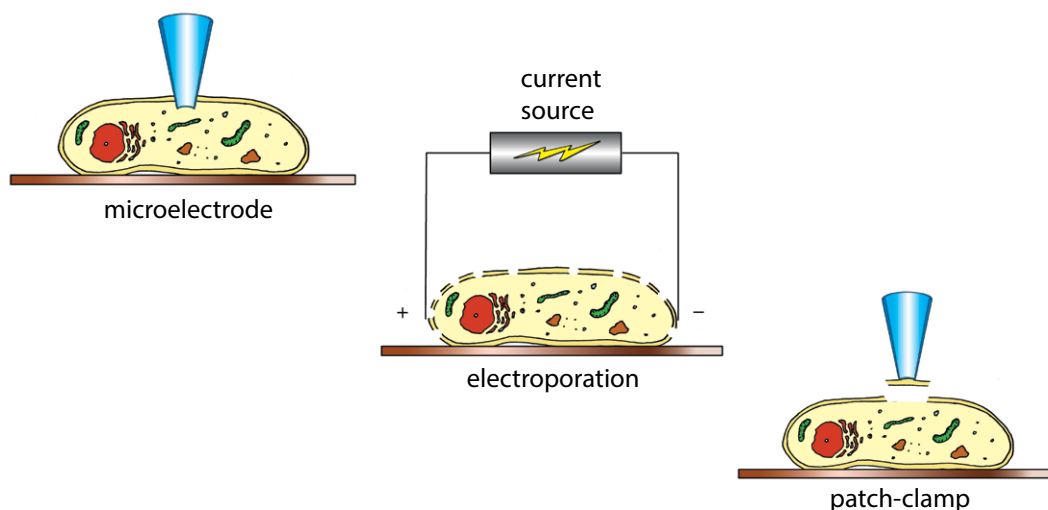
Stuart Taylor and colleagues have routinely used micropipettes to microinject calcium-sensitive dyes at multiple sites along the isolated muscle cell. Achieving this with a single micropipette requires repeated withdrawals that leave micron-sized holes. Yet for up to several days after injection, the cell continues to function normally (Taylor *et al.*, 1975).

The results of patch removal are similar. Here again, the hole in question is roughly a million times the size of the potassium ion. My colleagues, Guy Vassort and Leslie Tung, who routinely do these kinds of experiments, tell me that following removal of the 1- μm patch, the 10 μm isolated heart cell will frequently live on and continue beating.

A possible explanation for survival in all of these situations is that the wound rapidly reseals. With rapid healing, the deadly surge of ions could be stemmed. Residual membrane could be imagined to spread over the damaged zone as soap film spreads over the surface of water, covering the wound and thereby resealing the cell. By this “band-aid” mechanism, the wounded cell could be rescued.

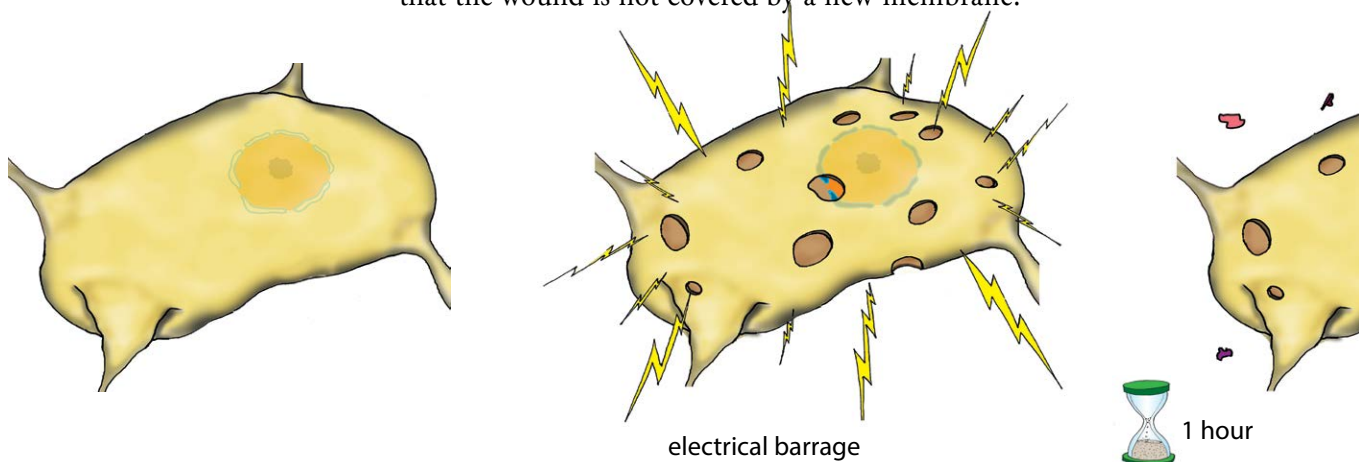
The difficulty with this otherwise attractive argument is that the mem-

Figure 2.1. Three methods of poking holes in the cell membrane.



brane is not at all like a soap film. The soap film may be many layers thick, and can spread by thinning. The membrane is a single lipid bilayer—it can spread only if constituent molecules spread. But X-ray diffraction (Korn, 1966) and fluorescence studies (Discher *et al.*, 1994) reveal a constant molecular packing density. Although molecules may diffuse within the bilayer, they cannot separate appreciably without causing membrane disruption. And if they could, it would still be necessary to find a reason why they should: what might induce the bilayer to spread?

In fact, the absence of resealing is confirmed by direct evidence. In the case of the withdrawn microelectrode, the hole can be seen either by direct microscopic observation (Nickels, 1970) or with even greater clarity if the withdrawing microelectrode leaves behind a deposit of electron-dense sediment (Nickels, 1970; 1971). The potential for resealing has also been examined in cut cells. Sectioned cells survive for use in electrophysiological studies, but electron micrographs show no evidence of membrane resealing, either in muscle cells (Cameron, 1988) or in giant axons (Krause *et al.*, 1994). Radical changes certainly ensue—exocytotic vesicle fusion may lead to some membrane replenishment (Bi *et al.*, 1995); and near the wound there may be a combination of vesicle accumulation (Krause *et al.*, 1994), cytoskeletal/vesicular coagulation (Fishman *et al.*, 1990; Spira *et al.*, 1993) and vacuole accumulation (Casademont *et al.*, 1988), but electron micrographic evidence confirms that the wound is not covered by a new membrane.



Long-term orifices are also confirmed during electroporation. Electroporation is a technique developed principally to transfer foreign molecules into cells (Fig. 2.2). Although permeability can sometimes be short-lived, this is not necessarily the rule. When molecules are introduced into the bath well after the end of the electrical barrage, entry into the cell is still possible. The window of opportunity depends on molecular size. In the case of huge molecules such as DNA, substantial penetration is observed when the molecules are added from 20 minutes to an hour after the shock terminates (Xie *et al.*, 1990; Klenchin *et al.*, 1991). Albumin, a common extracellular protein, can also penetrate for approximately one hour (Prausnitz *et al.*, 1994), and for smaller substances with molecular mass on the order of 1,000 Daltons, post-electroporation flow can occur for hours to days (Schwister and Deuticke, 1985; Serpersu *et al.*, 1985). Thus, the pores can remain open for long periods during which substances thousands of times the size of an ion can pass.

Clearly, then, wanton acts such as punching holes in the membrane do not necessarily wreak havoc within the cell. The holes are huge relative to the ion. Yet, there is no evidence that the cell really cares. In spite of stringent ion-concentration requirements, the cell can sail through such insults with little sign of any abnormal behavior.

If the examples above seem too technical, consider the behavior of the common alga *Caulerpa*, a single cell whose length can grow to several

Figure 2.2. Substances delivered substantially after the period of electroporation are able to enter the cell.



meters. This giant cell contains stem, roots, and leaves in one cellular unit undivided by internal walls or membranes (Jacobs, 1994). Although battered mercilessly by pounding waves and gnawed on relentlessly by hungry fish, this single cell cares not a whit; such breaches of integrity do not threaten its survival. In fact, deliberately cut sections of stem or leaf will grow back into entire cells. Severing the membrane is irrelevant.

Yet another example lies within the domain of experimental genetics, where otherwise genteel scientists will brutally slice innocent cells in two in order to monitor the fates of the respective fractions. When cultured epithelial cells are sectioned by a sharp micropipette, the non-nucleated fraction survives for 1-2 days while the nucleated, centrosome-containing fraction survives indefinitely and can go on to produce progeny (Maniotis and Schliwa, 1991). The cell does not seem to mind that it had just been sliced like a tomato. Sectioned muscle and nerve cells survive similarly (Yawo and Kuno, 1985; Casademont *et al.*, 1988; Krause *et al.*, 1984).

Then, there is the crawling cell that tears itself apart as it journeys onward (*see* Preface). Leaving behind a trail of cellular fragments is the rule rather than the exception (Chen, 1981). In the case of fibroblast cells, fragments down to 2% of initial volume continue to show standard behaviors such as ruffling, blebbing, filopodia production, and contact avoidance (Albrecht-Buehler, 1980). The fragments remain “alive” for up to eight hours.

Finally, and perhaps not surprisingly in light of all that has been said, ordinary mammalian cells are continually in a “wounded” state. Cells that suffer mechanical abrasion such as skin cells, gut endothelial cells and muscle cells are particularly prone to wounds—as confirmed by entry into the cell of large tracers that ordinarily fail to enter, such as serum albumin, horseradish peroxidase, and dextran (mol. wt. 10,000). Such wounded cells appear otherwise structurally and functionally normal (McNeil and Ito, 1990; McNeil and Steinhardt, 1998). The fraction of

wounded cells in different tissues is variable (Table 2.1). In cardiac muscle cells it is ~20%, but under isoproterenol stimulation the fraction rises to 60% (Clarke *et al.*, 1995). Thus, tears in the cell membrane are neither exotic nor arcane; they occur as a common event in normal, physiologically functioning tissue.

It appears we are stuck on the horns of a dilemma. If we insist that a continuous barrier envelops the cell, we need to reconcile the aforementioned series of observations and we need to explain why breaching the barrier is not more consequential than it seems to be. The anticipated collapse of ion gradients should quickly destroy cellular proteins (Choi, 1988; Berridge, 1994). On the other hand, if we concede that the barrier may not be continuous so that creating yet another opening makes little

Table 2.1. Cell wounding under physiological conditions (from McNeil and Steinhardt, 1997).		
Organ	Cell types	Percentage wounded
skeletal muscle	skeletal muscle cells	5-30
skin	epidermal cells	3-6
G.I. tract	epithelial cells	—
cardiac muscle	cardiac myocytes	20
aorta	endothelial cells	6.5
inner ear	auditory hair cells	—

difference, we then face an obstacle of a different nature. We challenge the long-held dogma of the continuous barrier, as well as the evidence on which the continuous barrier concept is presumably based.

Just how strong is such evidence?

MEMBRANE CONTINUITY?

The continuous phospholipid-membrane concept arose during the era of light microscopy. Because ordinary light microscopes cannot resolve structures on the order of 10 nm, what was identified as a membrane was probably the dense underlying cytoskeletal layer, which is 100 times thicker. The cytoskeleton is a subject of significance, to which considerable attention will be devoted (Chapter 10). The fact that it may appear continuous in the light microscope implies nothing about molecular scale continuity—or indeed whether the unseen lipid-bilayer might be continuous.

With time, the continuous phospholipid-membrane concept nevertheless drew broad support. Lipid arrays formed spherical vesicles that seemed analogous to cell membranes. Electron microscopy revealed the cell periphery to have a characteristically trilaminar structure that was inferred to correspond to the lipid bilayer. And freeze-fracture images showed striking images of the membrane's faces. What had been inferred early on from the light microscope seemed all but confirmed by these methods.

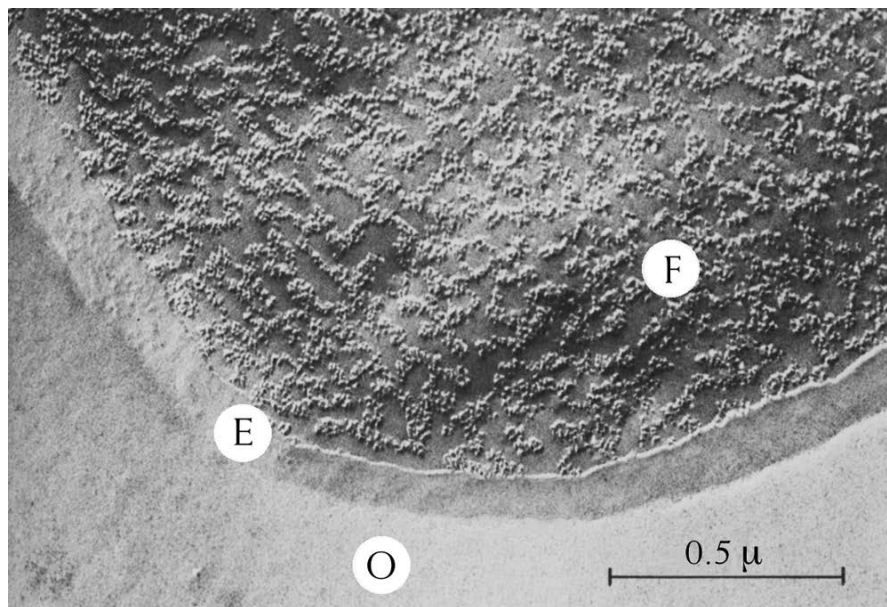
At the same time, it was becoming clear that the cell membrane was by no means phospholipid alone. With the isolation of membrane-based proteins, it became clear that the protein content of the membrane was appreciable. The current textbook estimate for typical membranes is on the order of 50%. In the inner membranes of mitochondria and chloroplasts, the ratio by weight of protein to lipid rises to 3:1, and in certain bacterial membranes the ratio is as high as 4:1 or 5:1 (Korn, 1966, Table 1).

Thus, the popular idea of a lipid bilayer studded with occasional proteins is misleading. The generic membrane is as much a protein structure with lipid inclusions as a lipid structure with protein inclusions. See Figure 2.3.

For some, this revelation may come as a surprise. The idea of a continuous phospholipid membrane is so deeply ingrained in current thinking that it is difficult to assimilate the fact that membranes can comprise 50% - 80% protein. I venture to say that if the protein had been identified before the lipid, the rubric of a protein membrane might have been adopted in place of the lipid membrane.

But the “protein” membrane begs the issue of continuity. Membrane proteins are typically large molecules that fold back upon themselves many times as they course through the membrane. The resulting crevices create natural flow pathways like cracks in concrete, through which water and ions can seep. These proteins impact nearby membrane-lipid molecules as well, altering their natural arrangement and creating additional ion-flow pathways

Figure 2.3. Disposition of proteins in the cell membrane. This red blood cell was quick-frozen, and then fractured in preparation for electron microscopy. The fractured surface (F) reveals many particles thought to be membrane proteins. The thin region (E) reveals the cell's exterior surface, while the region (O) is outside the fractured zone. After Pinto da Silva and Branton (1970).



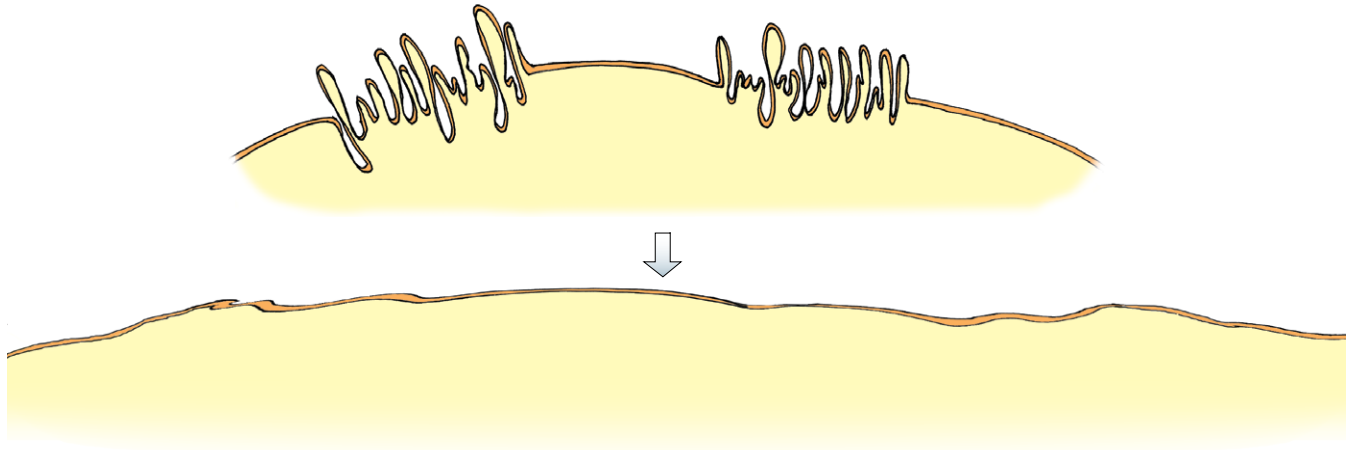
(Heimburg and Biltonen, 1996). Indeed, it is difficult to find any report of a protein stuck into a model membrane that does not increase permeability.

Given the leakiness of the protein-dominated membrane, it is worth reflecting on how some well-known observations that have seemed anomalous might now reconcile.

The first is an old experiment by a then-prominent physiologist who studied transmembrane potassium flux (Solomon, 1960). Flux was measured under control conditions and under conditions in which membrane lipids were partially removed. Potassium flux was the same—it did not depend on whether the lipids remained intact. The absence of physiological impact led the author to question whether the membrane is “superfluous to the maintenance of cellular integrity and intracellular function.”

Another well-known observation is on the transmembrane flux of water. Exchange of water through a lipid bilayer is extremely slow. Exchange through the cell boundary is an order of magnitude faster at least, and this excess has been explained by invoking specialized water channels that penetrate the bilayer (Dempster *et al.*, 1992). But water channels are superfluous within the discontinuous barrier framework; water can exchange naturally in the context of protein hydration (Chapter 4).

Another observation easier to accommodate with the protein-dominant membrane is a change of cell-surface area. In response to acute osmotic challenge, a doubling of membrane-surface area is not unusual in some cells. How such a change could be accommodated by a membrane has remained unclear, given that molecular packing undergoes no measurable change in the lipid bilayer even when the membrane is strained almost to the point of fracture (Discher *et al.*, 1994). In a membrane replete with convoluted, accordion-like proteins, on the other hand, accommodating such surface-area change is less of an issue; the proteins simply unfold (Fig. 2.4).



While these explanations may make sense, one is left with an uneasy feeling of having seen electron micrographs showing a continuous membrane surrounding the cell. Can such evidence be ignored? We are conditioned to interpret the trilaminar border that bounds the cell as phospholipid. Yet, when all lipid in mitochondrial membranes is dissolved by acetone treatment, the border persists (Fleischer *et al.*, 1965). And membranes of *E. Coli B* have a trilaminar structure even though they contain essentially no unsaturated fatty acids—ordinarily a major membrane-lipid component (van Iterson, 1965). Given these facts, one must wonder whether the trilaminar structure seen in the electron microscope can really represent the membrane phospholipid, or whether instead it represents some cell-interfacial feature highlighted by the staining procedure.

This issue is given much attention in a review by Hillman (1994). One of several questions raised by Hillman is why the trilaminar cell borders seen in electron micrographs are not replete with channel, pump, and receptor proteins. Nominally 20 to 30 nm in size, such proteins are larger than the 10-nm trilaminar border, and if they are as abundant as anticipated, they ought to show up either as stained blobs, or if they do not take up stain, as gaps. Yet the trilaminar border is almost always uninterrupted. What can this mean?

Figure 2.4. Protein unfolding can increase membrane-surface area.

Finally, if the membrane does not function as a continuous ion barrier, one may rightly ask why it should be there to begin with. I can think of at least three possible roles (Chapter 16): a scaffold into which membrane proteins are inserted; a partial barrier to retard the loss of soluble proteins and metabolites from the cell; and a deflector of transmembrane ion flow to those (protein) regions where useful triggering action can take place.

In sum, the view that the cell is surrounded by a continuous ion barrier does not seem well substantiated. The membrane is dominated by proteins and glycoproteins, which are able to exchange water, salts, and other small solutes. The barrier is leaky. Leakiness may explain the documented passive diffusion into the cell of peptides < 100 amino acids in size (Lindgren *et al.*, 2000). I hope this conclusion is not misinterpreted as implying that the membrane is unimportant, or that there is no membrane. The cell evidently does have a membrane—it is just that the role of this organelle may be different from the one that is ordinarily envisioned.

CONCLUSION

Continuing to move boldly, we took it upon ourselves in this chapter to reconsider the notion of the continuous ion barrier. If the barrier were continuous, we reasoned, violating its continuity by tearing large holes should allow ions to surge across the cell boundary and solutes to leak out, dramatically altering the cell's makeup, shutting down cell function, and eventually killing the cell.

But that did not happen. Whether created by shoving a micropipette into the cell, plucking a patch from the membrane, riddling the membrane with an electrical barrage, or slicing the cell into two, the wounds seemed to matter little; the cell could often continue to function as though there had been no violation. It was as though function could be sustained by the cytoplasm alone.

To gain perspective on this unsettling result we considered the evidence thought to underlie the barrier's presumptive continuity and found it to be less than certain. We noted that the lipid bilayer contained an abundance of proteins, which confer leakiness on the membrane. With a leaky nature, one can rationalize why poking another hole might not matter much.

The leaky membrane paradigm also lends understanding to the difficulties with channels and pumps. These elements arose to provide needed pathways for ions to flow through a membrane that was presumed impermeant. With permeance, such elements become redundant. Why their imputed function might seem out of accord with evidence is understandable.

Perhaps it is becoming clear that excavating toward the core of truth requires the peeling back of multiple layers of assumption. We are not yet through. The next chapter considers a layer of assumption even more fundamental than those considered—the assumption that led to the continuous barrier notion in the first place. Why was it necessary to postulate a continuous barrier?

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