BOT 4503
6 hours
Membrane Transport

Objectives

1. In simple terms, explain the function of biological membranes.

2. Describe an experiment and the results that would allow you to conclude that uptake of an uncharged species is a simple diffusional process. Plot v (rate of accumulation) vs. (concentration difference between the inside and the outside for an uncharged species) for simple diffusion. . . . for a transport-protein mediated process.

3. Briefly describe the three types of transport proteins, and explain their distinguishing characteristics. Of these, which are likely to saturate at only very high concentrations, above the physiological range? Of these, which are most likely to be limited by the rate of diffusion to the protein?

4. Give a rigorous definition of diffusion.

5. Name assumptions made in the derivation of the Michaelis-Menten equation. Define v, S, V_{max}, K_M. Make a v vs. S plot for a Michaelin process (either an enzyme, or a transport protein.) Label V_{max} and K_M. Know the familiar form of the equation and be able to use it in the description of a process. Work an example: let the $V_{max} = 1$ (i.e., 100% of anything); let the $K_M = 1$ (again, 100% of any value). Now, plot v as a function of S = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8. Make another plot; this time, let your S scale go from 0.5 to 6, and increment by 1.0. Make other plots: on the same graph, let V_{max} for experimental condition #1 change from 0.3 to 3.0, incrementing by 0.5. On another graph, let S change from 0.3 to 3.0, again incrementing by 0.5. Write an essay about your conclusions.

6. Compare the energetic cost of accumulating one mole of glucose from the outside (0.1 mM) to the inside (10 mM) with the yield of metabolizing it to carbon dioxide and water. Repeat this comparison with the outside being 1 mM and the inside being 100 mM. Now, let the outside be 0.1 mM and the inside, 100 mM. At what point will the cost of taking up glucose be prohibitively "expensive?"

7. What is a typical value for the plasmalemma electrical potential? What is the diffusion potential? . . . how does it compare with the overall membrane potential? How might a diffusion potential originate? (Your answer can be contrived, made just for the sake of demonstrating that you understand what a diffusion potential is.) What is the electrogenic component of the membrane potential? Give an example of how it might originate. How can you differentiate the diffusion component of a membrane potential from the electrogenic component? Does the diffusion potential have a direct energy requirement?

8. Why is the driving force associated with the membrane potential so large—after all, the actual voltage difference is only 0.001x that of household power supply?

9. In terms of the number of ions, what is a typical asymmetry across a biological membrane?

10. Know the Nernst equation and how to use it. Which is important, the concentration difference, or the concentration ratio (in a comparison of the inside and outside)? Is the membrane potential an attribute of the system or of the particular ion under consideration? In other words, is there a particular membrane potential for each ion? What about for the Nernst potential—is there only one for all ions, or does each ion have a particular Nernst potential, according to its own relative concentrations, charge, and valence? Explain what is meant by the expression, "60 mV will support a decade of (monovalent) ion asymmetry?" Do some sample problems (from my notes and from your text).

11. Name the three types of ATP-dependent H^+ pumps. Where are these pumps located? Describe two ways that the P-type ATPase can be distinguished from the others (phosphorylated intermediate? number and heterogeneity of subunits?) Why is the P-type pump also called an E_1E_2 pump? Give examples of P-type pumps in addition to the plasmalemma pump. How similar are these P-type pumps to each other in overall sequence homology? . . . homology in the phosphorylated domain? How can you easily distinguish the E_1P state from the E_2P state? Are these differences owing to conformational changes or to covalent changes? Do the two states differ in free energy? What is the stoichiometry of the pump? Describe experiments and theory to support this statement. Give reasons that earlier workers thought that this pump carried potassium. Give reasons that we now know that this pump does not carry potassium.

12. What is a hydropathy plot? How is it used to assign location to various spans of a membrane protein?

13. Plants have a unique proton pump. What "fuels" this pump and where is the pump located?

14. Describe/compare the "hopping" mechanism of proton translocation to the "alternating access" mechanism. What is a pK? Describe in outline how changes in pK could explain the requisite changes in affinity of the binding site on the "receiving" side of the membrane, compared with the "accepting" side of the membrane.

15. Discuss the chemiosmotic theory. If need be, refer to your BSC 2010 notes or text.

16. In simple terms, distinguish carriers from channels on the bases of speed of transport and mechanism of transport.

17. Define active transport, primary active transport, secondary active transport, facilitated diffusion, and simple diffusion. Which carriers are capable of which type of transport?

18. Write an essay on the integrated functioning of proton pumps and the uptake of sucrose—all the way from outside the cell to the vacuole. Explain how this whole process is directly or indirectly driven by the free energy of hydrolysis of ATP.

Lecture

Please refer to your text or other source, such as BSC 2010 notes, for general comments pertaining to the chemical structure of membranes and their function.

The essence of life is contained within membranes. Indeed, it is only with small risk of hyperbole that one asserts that membranes separate the living from the non-living—within the membrane-bound space, the chemical and ionic environment is regulated and is quite different from the environs. As an example familiar to everyone, mammalian cells accumulate potassium and exclude sodium. In fact, the maintenance of the "life" space is expensive, and estimates are that a plant expends about 10% of its energy budget on membrane functions. In addition to ions, such as sodium, that must be regulated, the traffic of an array of other substances, such as sucrose for nutrition, or calcium ion (an inter- and intracellular metabolic regulator) is controlled more or less precisely. A first question in a discussion of transport is to identify the nature of the transport: Does Substance A move through the membrane by diffusion through the lipid bilayer, or are special transport proteins involved?

Overhead: Diffusion or Not?

The rate of diffusion, recall, is proportional to the concentration gradient (of the neutral diffusing species). This general case is observed in the top panel. In brief, one can submerge or perfuse a tissue in different media that covers a concentration range of Substance A. If the rate of uptake across the membrane (which can be followed by a radioactive "tracer" or other means) is linear, diffusion may be assumed.

Uptake that is mediated by a transport protein in the membrane is characterized by saturation kinetics. As the bottom panel shows, at the lower end of the relevant concentration range, uptake may be linear or near linear. At high concentrations, however, increasing the external concentration will not result in increased uptake. A number of factors, which we will discuss later, will cause saturation kinetics. Now, simply consider a finite number of transport proteins in the membrane. At some high external concentration, the transport proteins that specifically conduct Substance A will **all** be occupied, so that additional "available" molecules of A will not result in an increase in the uptake of A. As shown to the right of the figure, we will consider three classes of transport proteins: (1) channels, which are molecular gates in the membrane. Typically, channels show saturation kinetics only at high external concentrations of

the permeating species, e.g., at 600 mM, (2) carriers, and (3) pumps, transporters that are fueled by the hydrolysis of ATP or the equivalent , which you have encountered in BSC 2010, with the example there usually being the sodium/potassium pump of animals.

Obviously, diffusion of the particular species under consideration is energetically "downhill," i.e., the movement is a spontaneous process, or, said equivalently, represents a loss of free energy. Uptake by transporter proteins may or may not be energetically downhill. We will come back to this idea later.

Overhead: Michaelis-Menten Model

A great deal of effort has been expended to understand protein-mediated catalysis (i.e., enzymatic reactions) and protein-mediated transport processes. The Michaelis-Menten Model often adequately fits ("explains") the empirical data obtained by studying the reaction rates (transport rates) as a function of the concentration of the substrate for the enzyme or transporter protein. (If the data do not "fit," models other than the Michaelis-Menten one are invoked, but we will not discuss complications.)

In the discussion of the overhead, I will simply describe the enzymatic case, where a single substrate molecule, denoted S, is converted to a product molecule, P. In the transport case, consider that S is the transport species on one side of the membrane, and P is the species on the receiving side of the membrane. **Eqn 1:** The free enzyme E is present (in molar quantities) at a concentration far far below the free substrate S. E and S are brought together by random collisions; these entities have an affinity with one another, and thus "stick," to form the ES, or enzyme-substrate complex. The rate of formation of this complex has a characteristic rate constant, denoted k1. (By "characteristic," I mean for a given process and under the given conditions, e.g., of temperature, pH, &c.) ES formation is reversible, with a rate constant k2. Substrate conversion to free product is irreversible, being overall described by the rate constant kcat. **Eqn 2:** The velocity is the product, kcat X [ES]. (The brackets indicate "concentration of . . . .") **Eqn 3:** The reaction is studied at steady-state, which means that all the concentrations of the various constituents are effectively unchanged during the considered period. As this statement applies to ES, the rate of formation is set equal to the rate of breakdown. **Eqn 4:** This equation is simply a statement of conservation. **Eqn 5:** This equation is obtained by division of equation 3 by (k1 X [S]). **Eqn 6:** This equation is obtained by substituting the expression for [E] (eqn 5) into eqn 4. **Eqn 7:** This equation is obtained by "collection of terms" in eqn 6. **Eqn 8:** The definition of the Michaelis constant, K_M, is given. **Eqn 9:** K_M is substituted into eqn 7. **Eqn 10:** This equation is a rearrangement of equation 9. **Eqn 11:** The velocity or overall

consumption of S is given by eqn 2; eqn 10 is multiplied by the rate constant kcat. **Eqn 12:** The maximum velocity, V_{max}, is obtained when ES=Etot. **Eqn 13:** This is the familiar form of the Michaelis-Menten equation. *You do not need to know how to derive this equation as we have done on the overhead but you do need to understand the logic. You also need to know ("memorize") the familiar form of the equation and be able to use it*.

Study the Michaelis-Menten equation. Particularly, note that if $K_M = [S]$, $v = V_{max}/2$. K_M has concentration units. It is important—from my perspective, at least—that you think of the K_M as being a shorthand way to describe a kinetics experiment. At the expense of redundancy, consider that you have measured the velocity of enzyme X as a function of various S concentrations, and given fit to the equation, you can completely describe the entire graph by simply stating the K_M and the V_{max}. It is not correct, as our sometimes-imprecise speech allows the neophyte to infer, that the K_M is an attribute of the enzyme itself. If one changes the experimental parameters, e.g., the pH, a tremendously different K_M may obtain. We will discuss K_M not only in transport processes, but also in many different contexts—ranging from the pH-stat mechanism to CO_2 fixation by whole leaves, so it is VERY important to learn it now.

What does the K_M tell us? First (and what most physiologists think of): if kcat<<k2, the $K_M = [E][S]/[ES]$, which is the dissociation constant of the enzyme-substrate complex (see eqn 5). In other words, K_M is the affinity of the substrate with the enzyme. (In technical speech, one often hears affinity "for," but "with," "between," or some other preposition that recognizes the mutual relationship is preferred, in my (personal) opinion.) What does the V_{max} tell us? First: how fast the catalysis can occur. Together, the K_M and the V_{max} (for enzymes that fit the model) provide a very useful way of predicting a reaction rate. BOTH must be considered. Play with the equation: If km>>[S], the reaction rate will be slow, even if V_{max} is large. If $[S]>>K_M$, addition of more S will not speed the reaction.

As mentioned, the Michaelis-Menten Model does not fit all cases, such as (1) "perfect" enzymes, where the rate of catalysis or transport is limited by diffusion. This limitation sets the final upper bound for rate. (2) enzymes that have more than one active site (where the E and S interact) if formation of ES_i affects binding of other S_j to different active site(s). (3) in some cases, enzymes that are inhibited or activated by various effectors. (4) any interaction that does not fit the assumptions of the model.

For uncharged chemical species, we have already learned that a species diffuses from a region of high concentration to a region of low concentration, and we applied that idea to the movement of gaseous water. The following overhead permits a quantitative assessment of the energy cost (or yield):

Overhead: Energetics (uncharged species)

The top half of this overhead shows the familiar equation that quantifies the energy "stored" if an uncharged chemical is at higher concentration on one side of a membrane. Look at the form of the equation: concentration is expressed as a ratio (i.e., the ln is a dimensionless number). Said another way, the equation is blind to the actual magnitude of the concentrations. In other words, if the concentration outside is 0.1 mM and the concentration inside is 1 mM, the ratio is 10, and computes exactly the same as if the concentration inside were 1 M and the concentration inside were 10 M. The take-home message is RATIO.

As stated, the equation permits calculation of the energy required to accumulate a species against a concentration difference (or the energy yield associated with the relaxation of a concentration difference). As shown, movement of 1 mol of glucose against a 100x-concentration difference "costs" 2667 calories. The bottom half of the overhead permits one to place this cost in perspective. The complete combustion of glucose to carbon dioxide and water yields 673,000 $cal \cdot mol^{-1}$. As you know, metabolism does not proceed under the standard conditions (unit molar quantities, STP) nor does metabolism proceed at 100% efficiency. Notwithstanding, one easily infers that the specific direct cost of accumulating glucose will be repaid many times by the energy yield in metabolizing it. (Other costs not mentioned are those of maintaining the genome, synthesizing and placing the transporter, the inefficiency of "fueling" the transporter, the inevitable imperfection in membrane permeability and leakage.)

To consider the direction and magnitude of the driving force for the movement of a charged species, the membrane potential must come into play. Therefore, we turn briefly to describe the origin of membrane potentials. (In this description, we will ignore a third cause, which is not a significant component of the membrane potential of plants.)

Overhead: Origin of Membrane Potentials

Diffusion Potential, shown at the top, results from the differential permeability of anions and cations. Consider an initial state in which all K^+ and all Cl^- are on one side of the membrane. Over time, the ions collide with the membrane. Chloride is more often reflected by the membrane than is potassium. At the time of observation, there are more + charges and fewer -charges on one side of the membrane than on the

other. This asymmetric distribution of charges gives rise to the major membrane component. One notes that there is no direct energetic requirement to generate this potential (but the order associated with the initial state was "expensive" to create—KCl "does not want" to be at high concentration on one side of the membrane and at zero concentration on the other side of the neutral membrane. Speaking in the vernacular, at some point, the "desire" of K^+ to go to the region of low concentration will be balanced by the "pulling power" of the electrically negative region. I.e., the membrane potential—an attribute of the system—will affect each ion, but in different ways. By way of example, Cl^- will "want" to cross the membrane for the additional reason that such movement would transfer it to an electrically positive area. (Recall that electrical potentials are differences between two regions, and that electrical potential can not be expressed as an absolute quantity—there must be a reference state.) At the end of time, of course, all species will be at the same concentration throughout the entire system and the membrane potential will have disappeared. Thus, although maintenance of the diffusion potential does not have a direct energy requirement, energy must be expended to create the concentration difference and to maintain the conditions that will cause diffusion. Although the diffusion potential is large relative to the other major source of the overall potential and represents a large driving force (because of the thinness of the membrane), the diffusion potential does NOT represent much charge asymmetry--for each 10^5 to 10^6 positive charges, there is only one more negative charge on the inside!

The second major component of the membrane potential of plants is from the electrogenic pumping of ions. The prototypical ion pump in plants is the Mg-dependent, potassium-activated P-type proton-extruding ATPase on the plasmalemma, about which you will hear a great deal over the next hours. At this time, it is sufficient to learn two salient features: (1) This transporter protein is electrogenic—i.e., its activity results in net charge transfer, in this case, one + charge is pumped out (carried by a proton, or, and I will hereafter desist, the hydronium ion). A relatively new word that is being applied to ATPases that are electrogenic is "electroenzyme"—we'll have to wait to see if this word catches on. (2) Each charge transfer has a direct requirement for ATP. Thus, in contrast to the diffusion potential, which runs down slowly, the electrogenic component of the membranes disappears immediately if the source of ATP (cellular metabolism) is stalled by application of a poison.

Overhead: Energetics (Charged Species)

As mentioned *ad naseum*, the direction and magnitude of the driving force for the movement of a charged species is a combination of the concentration difference of that particular ion in the two regions and the membrane potential difference of the two regions of the system. "Concentration" applies to the ion under consideration, whereas "membrane potential" is an attribute of the system. (As an aside, we have not considered hydrostatic pressure differences, because they have little effect; by way of example, a relatively modest membrane potential difference of -100 mV could be offset by pressure, but the pressure required would be ca. 3200 atmospheres!) Fortunately, it is straightforward, if requiring attention, to calculate the interaction of concentrations and electrical differences in consideration of driving forces. The Nernst equation is a "what if" equation—e.g., **what** would be membrane potential be if the *i*th ion is at such and such concentration on the respective sides of the membrane and **if** the electrochemical potential of the *i*th ion is the same on both sides of the membrane. The equation has four components that you need to keep in mind: concentration (out), concentration (in), the charge of the ion, and the membrane potential required to "support" the concentration RATIO difference.

(The Nernst equation was derived by using the assumption that the *i*th ion is at electrochemical potential equilibrium—it does not allow you to calculate the energy cost (or yield) associated with transfer of a charged species. As you infer, the derivation of the Nernst equation SET the ΔG for movement at zero. The Nernst equation—for calculation of the direction and magnitude of the driving force for movement of a charged species—does have the same form as the ΔG associated with the transfer of an uncharged species between regions of different concentrations. We will not calculate the energy associated with charge transfer, but we will describe some consequences of such calculations.)

In the following, we will do a couple of example calculations:

Overhead: Energetics (Charged Species, contd)

The data in the chart were taken from measurements on a giant algal cell (which is large and thus permits the facile collection of cytoplasmic contents). These data are not representative of a higher plant cell, but they are simply used to demonstrate how a calculation may be done. Several questions, permutations of the same concept, are given on the overhead. The Nernst equation, recall, was derived on the assumption that the *i*th ion is at equilibrium on each side of the membrane. We may restate the question in several ways: given the concentration asymmetry, what would be the "balancing" membrane potential. This value is, of course, the Nernst Potential, which is different for each ion. Consider K^+. Plugging the

concentration values into the equation, one calculates that the balancing potential would be -173 mV. The observed membrane potential is only -116 mV, thus the driving force is +58 mV (to the outside). The actual bookkeeping of the signs (+ or -) and interpretation (does + mean go to the outside?) is at best a nuisance and at worst a mistake, so simply your life. Examine the equation and the system under consideration. First, recognize that if the ratio is flipped, that simply changes sign. Thus, as a matter of convenience, you can forget whether concentration (outside) is the denominator or the numerator. Second, recognize the log-base$_{10}$ formulation. That means that one decade of concentration difference simplifies to the coefficient, nominally 60/z. As an example, a monovalent cation, such as K^+, is at equilibrium if the concentration ratio is 10 (log 10 = 1) and the membrane potential is 60 mV; the required easy part is to supply a little logic—if the concentration on the inside is higher, then the potential must be negative internally (to "hold" the positive charge). . . . again, for K^+, if the concentration is 100x higher inside, what would be the Nernst potential? Simple, (60/1)log (100) = 60 x 2 = 120 and you know that the units are mV, and the sign is negative. Or, you can, of course, keep track: (60/+1)log (0.01) = -120 mV. . . . and a 1000x ratio would be 180 mv. As z = +2 for a divalent cation such as Mg^{2+}, the coefficient reduces to +30 mv. As z = -1 for a monovalent anion such as chloride, the coefficient reduces to -60 mv. Finally, and apparent, all plasmalemma potentials of intact unperturbed cells are negative (with respect to the outside, which implies that at electrochemical potential equilibrium, cations should be more concentrated on the inside, and anions, on the outside.

For the case on the overhead, assuming the system to be at steady state (the membrane potential and the concentrations stable), it is apparent that the driving force is and has been for potassium to move to the outside of the cell. In other words, potassium did not reach such a high internal concentration by diffusion alone. Some work must have been performed on potassium to move it from a region of lower electrochemical potential (outside) to a region of higher electrochemical potential (inside). Holding the assumptions, we can announce unequivocally that potassium uptake was not passive, a special word, especially historically, to denote that energy input is not directly required for uptake. (The antonym of passive is active.) The actual membrane potential would support an asymmetry of 100x (i.e. -116 mV = 60(log 0.01) mV. Thus, if the Nernst potential were the same as the membrane potential, the inside concentration would be 10 mM (0.1 mM x 100). Thus, we know that most internal potassium (ca. 90 % = (93-10)/93) was accumulated against the electrical chemical potential. The situation with regard to Na^+ is different: the Nernst potential is actually less negative than the observed membrane potential. Thus, it is possible that Na^+ moved into the cell passively. Of course, only based on the energetics, we can not infer the actual mechanism, but we do know that we do not need to invoke active transport.

As mentioned in the beginning of this lecture series, there are three types of transporter proteins in membranes that facilitate the movement of individual molecules or ions from one side of the membrane to the other. (A different type of transport, endocytosis, will not be covered. This type of transport, as you know, involves the inclusion of a bulk phase of external media by the invagination of the membrane.) Actually, these transporters do not function separately, but they work together. First, we will look at the transporters individually.

A first type of transporter is the pump, which is powered by ATP or an equivalent. There are several types of pumps. In BSC 2010, typically, the Na/K-ATPase is discussed in fair detail. There is no exact equivalent in plant cells. (Indeed, except for a special group of plants, Na^+ is not a required element.) You may have discussed a Ca^{2+}-ATPase when muscle function was covered in BSC 2010. Plants have a Ca^{2+}-pump also, but we will not cover it. Our focus will be on the proton-extruding magnesium-dependent potassium-stimulated ATP-powered pump on the plasma membrane of higher plant cells. This pump, which will be dubbed simply "ATPase" hereafter, is similar to the Ca^{2+}-pump, the Na/K-pump, the fungal H-pump, and certain others, because all these electroenzymes have a phosphorylated intermediate. (I.e., when the enzyme acts on ATP, ADP is released to solution, the Pi is not: it is covalently bound to the pump. After (or during) a cycle, the Pi is hydrolyzed from the enzyme.) Pumps that have a phosphorylated intermediate are called P-type ATPase. ("P" stands for phosphate, but in plants, it could just as easily stand for plasmalemma, as this is the only location for this type of pump.) Although P-type ATPases are only generally similar to each other in overall sequence homology (ca. 25 % at the amino-acid level), the sequence around the phosphorylation site is highly conserved. This observation suggest the antiquity of this type of pump in evolutionary terms (i.e., the simplest interpretation is that the common ancestor of plants and animals had the same progenitor pump.) Moreover, the conservation implies that such a pump is essential. Another characteristic of a P-type pump is that it exists in two forms: the E_1P form can react with ADP to form ATP; the E_2P form can not. Thus, P-type ATPases are also called E_1E_2 pumps to denote their *modus operandi*. At the risk of revealing the obvious, the two states differ in conformation (and free energy), but they do not differ in covalent structure.

Plants have other types of H-ATPases also. One, the so-called V-type, is found on the tonoplast. (You can remember "V" as "Vacuolar," but this type of pump is found also in lysosomes and coated vesicles.) The F-type[1] ATPase (or ATP synthase) is associated with chloroplasts, mitochondria, and prokaryotes. Neither of these other two types function by formation of a phosphorylated intermediate, and both of them comprise a collection of disparate subunits, whereas the ATPase of the plasmalemma has only

[1] F is an abbreviation for "coupling factor" because it couples the relaxation of a proton gradient with ATP synthesis. In the chloroplast, this ATPase is called the CF ATPase (for "chloroplast coupling factor.")

a single type of transport subunit (nominally 90 kD), one individual being the minimum catalytic unit (but *in vivo* measurements indicates that it exists as a dimer, and, more recent experimentation indicates that there is also a regulatory subunit). Plants have a unique proton pump in addition to the common types enumerated above: in the tonoplast is a proton pump that is powered by the hydrolysis of pyrophosphate. Conventional wisdom placed the pyrophosphate level in cells very, very low. This conventional wisdom is ensconced in current biochemistry texts, but it is wrong. Some scientists believe that this PPi-pump has a K^+ transport capability as well as its well-known ability to pump protons. Whereas we will not discuss these other types of proton pumps, a mention of them is necessary, if only to caution that we will focus only onthe P-type ATPase.

Biochemically, the ATPase has been rather well characterized, as implied by the foregoing sequence homology analysis. Knowing the sequence, one can examine the polar (or non-polar) nature of each amino acid. One finds that regions of the primary sequence are made of amino acids that are hydrophobic; other regions are hydrophilic. This analysis is the basis for a hydropathy plot. From this information, models detailing regions that interact with the aqueous phase and other regions that interact with the lipid phase of the membrane are constructed. Overall, the hydrophobic regions are "assigned" to transmembrane spans whereas the hydrophilic regions form loops on either side as the protein laces back and forth across the membrane.

Overhead: Topological Model for ATPase

As this overhead shows, the general arrangement of the ATPase across the membrane is established, although the COOH-terminal region assignment is equivocal. In addition to the membrane spanning regions that form the "channel," the phosphorylation site and the domains that catalyze its phosphorylation and dephosphorylation are assigned. (The use of the word "channel" here should not be confused with *bona fide* channels that will be covered later.)

As we have mentioned, several different kinds of pumps translocate various numbers of ions from one side of the membrane to the other. E.g., for each ATP hydrolyzed by the Na/K pump, five ions and a net of one charge are transported. With "our" ATPase, the stoichiometry is unity (one proton translocated for each ATP hydrolyzed). In brief, native vesicles prepared from plasmalemma were studied; the rate of pH change on the outside (corrected for membrane leakage) was correlated with the loss of ATP within the sealed vesicles. The energetics also imply that unit stoichiometry is the limit (put simply, for the overall

process to be spontaneous, the free energy loss associated with the hydrolysis of ATP must exceed the free energy increase associated with the transfer of a proton to the more acidic [as much as 3 pH units] cell-wall space).

We do not yet know how the ATPase transports the proton from inside the cell to the outside. I will present two models.

Overhead: Two mechanisms for proton translocation.

The first mechanism ("hopping") is based on the presence of a hypothetical proton wire structure. The proton wire structure is a path through the ATPase. The hypothetical path consists of hydrogen-bonded groups (R-groups of amino acids, and possibly bound water). For simplicity, one might consider that the path is rigid, as shown at the top of the overhead. As a matter of illustration, this path might be hydroxyl groups. One proton dislodges the next and so forth. This simple hopping is unlikely because of energetic considerations. A more complicated proton wire scheme is shown in the bottom two sequences. This mechanism involves the movement of protons between an H-bonded state and a covalent state AND conformation change of the protein. This first mechanism, regardless the precise way that it is implemented, implies that the bound proton is transported by proxy. I.e., over one cycle of ATP hydrolysis, one proton is taken up on the inside and one proton is released to the medium, but these are different protons. This "net" movement is in contrast to the second mechanism, explained below. Evaluation of the feasibility of this mechanism awaits elucidation of the 3-D structure, which permits localization of putative proton-wire structures, which could be disrupted by site-directed mutagenesis.

The second mechanism is referred to as the alternating site mechanism. In this mechanism, a proton is bound on the inside and the protein changes conformation in such a way that this same proton is exposed on the exterior of the cell and released there. To be workable, the affinity of the proton with the protein much be much higher in the more alkaline cell interior than the affinity of the proton with the protein when the proton is exposed on the exterior side, which is more acidic. There have been several suggestions as to the nature of the proton-binding site. The simplest only involves the protonation of an R-group of an amino acid. The protonation of a functional group like a carboxylate or an amino is, first, dependent on the pH. E.g., at pH 4.7, half of the generic carboxyls on acetic acid are COO^-, and the other half are COOH. At a lower pH, more are COOH; at a higher pH, more are COO^-. This halfway point is called the pK, a very useful term and concept. (The "precise" distribution or ratio of COO^-/COOH can be calculated easily from

the Henderson-Hasselbalch equation.) pKs, however, are not fixed, even for a particular functional group. The pK depends on temperature and ionic strength, e.g., and also on the chemical environment (the three carboxyl groups on citrate have quite different pKs). The pK also depends on bond angle or strain. Thus, it is possible to consider a functional group with a low pK on the inside, where it would "pick up" a proton; when this functional group is exposed to the exterior, it might be supposed that pK would be increased, and the proton released. More recently, more complex interactions, supposing that H^+ (as the hydronium ion) acts as a coordination center have been proposed.

We do not yet know how the scalar process of ATP hydrolysis is coupled to the vectorial process of proton extrusion against an electrochemical potential gradient. A center of focus is the conformation changes that are involved in the cycle of phosphoenzyme formation and breakdown.

Finally, we can speculate on the role of K^+ ion on the activity of the pump. From the beginning, this stimulation by potassium was recognized. Logically, at the time, the pioneers in this work felt that the pump moved a proton to the exterior at the same that it caused uptake of a potassium ion. There were several reasons for this incorrect theory; e.g., if the pump acted on K^+, the pump would certainly be stimulated by its substrate. Moreover, the specificity of ion uptake by the plasmalemma ($K^+ > Rb^+$ &c.) matched the sequence specificity of ATPase stimulation. It is now well established that proton extrusion and potassium uptake are not *directly* coupled events: stimulation is fairly small, it is possible to measure proton pumping in the absence of K^+, and studies with inside-out vesicles showed that potassium stimulated the ATPase on the cytoplasmic side (whereas, if it were related to transport, one would expect stimulation from the site of uptake, and not release!). As shown earlier, the current model calls for K^+ stimulation of conformation change and release of Pi from the protein.

What does the proton pump do and why is it important? Obviously, the pump can serve to alter cellular pH, i.e., to regulate to optimum against proton-releasing reactions in the cell. This is often referred to as coarse pH control, and other mechanisms account for fine control. Important though it may be, let us set aside that idea to focus, instead, on one of the most fundamental biological concepts that were developed in the 20th century. THE ENERGY RELEASED BY ATP HYDROLYSIS CAN BE CONVERTED INTO THE ENERGY REPRESENTED BY THE DIFFERENCE IN ELECTROCHEMICAL POTENTIAL OF AN ION ACROSS A MEMBRANE or THE ENERGY RELEASED BY THE RELAXATION OF AN ELECTROCHEMICAL POTENTIAL DIFFERENCE ACROSS A MEMBRANE CAN BE CONSERVED IN THE PHOSPHORYLATION OF ADP. Thus, the relaxation of a proton gradient across the mitochondrial or chloroplast membrane, mediated by the F-type ATPase (ATP synthetase) is coupled with ATP formation. Though different in stoichiometry, this reaction is reversed at the plasmalemma. Said redundantly, pumping out protons simultaneously alters two

parameters: (1) elevation of proton concentration itself in the apoplast, and (2) hyperpolarization ("makes more negative") of the membrane potential. As will be seen, protein-mediated influx of protons (**down** their electrochemical potential gradient, because of concentration of protons and the sign of the membrane potential) may be inextricably linked to the influx of a second solute, e.g., sucrose, which moves **against** its chemical potential gradient. As long as there is an overall loss of free energy, transport will proceed via these carriers. As will also be seen, uptake may proceed *via* molecular gates in the membrane. Movement through these gates is a spontaneous process, i.e., down an electrochemical potential gradient. As the proton pump hyperpolarizes the membrane, the pump creates or strengthens the driving force for cation uptake through channels.

We turn our attention now to carriers and channels, of which there are many kinds. First, we will distinguish carriers from channels. A carrier operates by selectively binding one or more types of ions or molecules on one side of a membrane and delivering the transport species to the other. Said another way, carriers have a binding site that may face either side of the membrane. That is, in this way, they operate like the "alternating access model" that we just reviewed for the pump. A channel is generally less selective: a channel is an aqueous continuum from one side of the membrane to the other. As you may infer, carriers are relatively slow (10 to 10^4 molecules per second) and channels are relatively fast (10^6 to 10^7 molecules per second.) In fact, the speed with which channels conduct may be limited only by the rate of diffusion, saturation kinetics may not be observed over the physiological range.

We have reached the point at which we need to expand and clarify definitions. **Active** transport is transport against a chemical potential gradient or an electrochemical potential gradient, in the case of charged species. Recall that the proton-extruding ATPase transported protons against their concentration gradient (from about pH 7 to about pH 5) AND from a region of more negative electrical potential. Thus, transport was certainly active, being driven by the free energy released on the hydrolysis of ATP. Transport that is active AND "powered" by alterations of covalent bonds (e.g., ATP $\rightarrow$ ADP + Pi) is referred to as **primary** active transport. **Secondary** active transport is also transport against a (electro)chemical potential gradient, but in this case, the transport is not linked to the breakage of a covalent bond. Instead, transport is linked to the flux of another molecular species that is moving down its energy gradient. By way of example, and taking the liberty of criminal speech: consider that sucrose is at low concentration outside the cell and at higher concentration inside the cell—thus, sucrose does not "want" to move into the cell. On the other hand, the electrochemical potential of protons is much higher outside the cell and, thus, protons "want very badly" to move into the cell. The sucrose carrier on the plasmalemma will link the transport of sucrose and protons into the cells. Thus, sucrose uptake is driven by the loss of free energy associated with the influx of protons. An important point is that sugar and amino acid uptake

by plant cells is via secondary active transport mechanisms. Indirectly, uptake of sugar and of amino acids requires ATP, but the carrier itself does not. (You may wish, again, to draw an analogy with the Na-driven glucose transporter in animal cells that you learned about in BSC 2010.) Transport that occurs down a (electro)chemical potential gradient is **passive**. Recall that the definition of diffusion was net migration of a substance under the influence of a (electro)chemical potential gradient. Thus, passive transport is a diffusional process. **Simple** diffusion (called on the first overhead of this series only "diffusion," as an uncomplicated way to introduce the subject) is diffusion through the lipid bilayer. Simple diffusion, then, is unlimited virtually, and is always proportional to the energy gradient. **Facilitated** diffusion is also down a potential energy gradient, the molecule moves across the membrane by a specific carrier or channel. Thus, as I have tried to "sneak" into our discussion, movement through channels is a diffusional process, but as channels are finite in number, channel-mediated transport (as well as carrier-mediated transport) can be saturated, albeit at very high concentrations. By way of review, all pumps operate by a primary active transport mechanism. Carriers function by either a secondary active transport mechanism or by a facilitated diffusion mechanism. Channels operate by a facilitated diffusion mechanism. (N.B. As we learn more about the molecular mechanisms of transport, we find that some of the historical terminology does not quiet fit; thus, the definitions above, being the most current might be at variance with tertiary literature. You are encouraged to learn the above, and to ignore other definitions.)

Overhead: Integrated function of carriers and pumps

This overhead, somewhat redundant with the preceding text, explains how pumps and carriers work together to cause the uptake of a substance against its potential energy gradient. On the right side, note that the energy of ATP hydrolysis is used to "elevate" H^+ to a higher potential energy, as we have said *ad naseum*. Then, sucrose is transported into the cell by linking that positive free energy change to the relaxation of the proton gradient. Overall, of course, for the process to occur spontaneously, the free energy change must be negative. (The free energy loss of H^+ translocation must be greater than the free energy gain of sucrose translocation.) In this case, at the plasmalemma, protons and sucrose are translocated in the same direction; thus, this sucrose transporter is a symport. (If the carrier only translocated sucrose inwardly, it would be a uniport, and it would operate by a facilitated diffusion mechanism.)

The situation is somewhat different at the tonoplast (left side of overhead). Again, the cytosol is electrically negative with respect to outside the cytosol (i.e., the vacuole). Again, the proton concentration is lower in the cytosol than outside the cytosol. Thus, the V-type ATPase (or, alternatively; the proton-

translocating pyrophosphatase) pumps protons into the vacuole against the electrical and chemical components of the electrochemical potential gradient. The difference here is that sucrose moves into the vacuole and this transport is linked to the efflux of protons. Thus, the sucrose carrier on the tonoplast is an antiport.

The sucrose symport is one of the best characterized carriers in plants. Bob Giaquinta first postulated its presence in the 1970's. Because of the central role of sucrose in plant metabolism (in a typical plant, about 80% of the carbon that is reduced to carbohydrate by photosynthesis is translocated out of the source leaf), a great deal of effort has been expended to characterize this protein. The activity of a proton-driven carrier can be inferred by the study of transport into or out of sealed vesicles. As protons are a substrate, the effect of pH can be studied along with the electrical properties (this carrier—like the proton pump—is electrogenic.). By such studies, it has been established that the stoichiometry is 1 H^+/1 sucrose.

Identification of a protein of low abundance (like most carriers are) is problematic if only standard biochemical techniques are employed. Fortunately, molecular biological techniques are more efficient at "fishing" out such proteins, though indirectly.

Overhead: Molecular cloning of the plasmalemma sucrose symport

Yeast cells can utilize sucrose as an energy source. This utilization may result from parallel pathways. As shown on the left, yeast cells can take up sucrose. Perhaps as only a semantic point now, the carrier is for maltose and sucrose is an alternative substrate. As shown on the right, yeast cells may excrete the enzyme invertase, which catalyzes the hydrolysis of sucrose to its constituent hexosyl moieties. Then, a different specific carrier takes up the hexose. A double mutant (lacking both the maltose carrier and the extracellular invertase) cannot live if supplied with only sucrose. The center panel of this overhead shows the textbook depiction of the preparation of a cDNA library. The normal plant tissue (which, of course, synthesizes the sucrose transporter) is extracted. The mRNA is reverse-transcribed to produce cDNA. (Copy DNA is the complement of the population of mRNAs in a cell at a given time.) A cDNA library is constructed—many copies of each cDNA species are separately made. Thus, one particular cDNA clone may encode for such-and-such enzyme in photosynthesis; another, for a different protein; and so forth. The bottom panel shows that each clone is "placed" into a yeast expression vector. If a particular transgenic yeast culture can grow on sucrose, the implication is that the cDNA with which it was "infected" coded for the plant sucrose translocator. This assumption was tested. First, sucrose uptake required a proton

electrochemical potential gradient. Adding so-called protonophores, chemicals that insert into biological membranes and allow the passage of protons, easily tests this for idea. After a short interval, protons are at electrochemical potential equilibrium across the membrane, and thus can not be used to drive secondary active transport.) Second, the yeast cells accumulated sucrose in a Michaelian fashion; under the experimental conditions, with a K_M of 1.5 mM. This kinetic attribute matched that of uptake of sucrose by the plant cells from which the cDNA was derived. In summary, the sucrose symporter is in the range of 55-62 kD (from various plants) and belongs to its unique class of sugar transport proteins, with only a distant relation to other transporters that can be classified into superfamilies. As inferred from hydropathy plots, the protein spans the membrane 12 times (six loops).

Channels are the last category of transport proteins that we will discuss. To restate the general situation, channels provide an aqueous transport pathway, and they support only facilitated diffusion of ions. More specifically, ion channels are proteins that catalyze rapid, passive, electrogenic uniport of ions through pores spanning an otherwise poorly permeable lipid bilayer. At the expense of redundancy, permeation of ions through channels can often be described adequately by simple diffusion through a water-filled pore long enough to traverse a lipid bilayer, but more complex models are often required to explain the data. It is too easy, however, to take this simple explanation too far. Channels can be selective. Some potassium channels permit the permeation of potassium at a rate of 10 000x the rate that they transmit sodium ions. (This is the more remarkable when one considers that the Na^+ ion is smaller than the K^+ ion, in the non-hydrated state, which is how they are supposed to travel.) The question is how can the channel be so selective **and** so fast. The consensus is that channels have multiple (three or more) specific binding sites within the pore "mouth." These sites bind say, potassium, with high affinity, and potassium ions go through the channel single-file. Biophysical studies suggest that the file of potassium ions are mutually repulsive and that this charge repulsion causes the affinity of potassium and the channel to lessen, so that the ion is released on the "receiving" side of the membrane. Generally, these channels are closed; they open in response to environmental or physiological signals. There are many, many different types of ion channels, and even many, many different types of channels for a particular ion within a particular organism or even cell type. Channels are involved in very, very different types of physiological processes. E.g., one type of potassium channel is involved in neuronal excitability and hormone release, and others are involved in the regulation of stomatal aperture size. In fact, there have been many reviews that focus only on potassium channels. Despite the diversity of channels and their physiological importance—the 1991 Nobel Prize was shared by Sakmann and Neher for their development of methods to study channels—they nevertheless are at relatively low abundance, a "biological average" being perhaps 1 potassium channel per square micrometer.

How can channels be distinguished, or what is the basis for the assertion that there are so many types? First, certain pharmacological reagents of which there are now many types in hand may selectively block channels. E.g., the scorpion toxin, the peptide charybdotoxin, blocks certain large conductance Ca^{2+}-dependent potassium channels by plugging the mouth of the pore. The smaller molecule, triethylammonium (TEA) blocks certain types of channels by blocking the pore itself. (This use of pharmacological reagents perhaps reminds you of the use of such compounds to distinguish ATPases.) Second, channels may be characterized by their selectivity. As noted, some channels distinguish between closely related ions, such as sodium and potassium. Other channels are quite nonselective. Third, channels may be distinguished by the direction in which they transport. Some are "inward rectifiers" —e.g., when the membrane potential is very negative, which drives K^+ uptake, the passage of K^+ into the cell drives the membrane potential toward zero. A potassium outward rectifier would work under opposite conditions. On the other hand, some channels allow permeation in either direction. Fourth, channels may be distinguished based on the speed of transport, conductance. Fifth, channels may be distinguished based on the speed of activation. As mentioned, certain conditions open channels. Some channels open immediately upon the signal, whereas others open only after a small delay ("slow-activating" vs. "fast activating"). Similarly, some channels close with a certain probability **even** though the stimulating condition persists! Sixth, the permissive condition for channel opening is often the basis for distinguishing a channel. As briefly alluded to, some ligands—in the earlier example, Ca^{2+}—cause channels to open (or close). Some channels open by conformation changes that are physically induced. These "stretch-activated" channels are known for plants, but you might be more immediately familiar with one that signals your "desire" to dash off to the tinkle-atorium. Importantly, some channels are voltage-regulated. Some open only when the membrane potential is more negative than a certain value. Thus, proton extrusion by the plasmalemma pump not only sets the thermodynamics to a condition favorable for potassium influx, but the voltage change also opens the molecular gates for potassium influx! (This is an important unifying concept.) Having finished the individual enumeration preceding, I emphasize that the channels may actually be regulated by several factors acting together. E.g., in one case, a potassium in-channel is voltage-gated at -100 mV. (The channel is closed until the membrane potential is more negative than -100 mV.) However, elevation of cytosolic Ca^{2+} from a nominal cytoplasmic resting level of 100 nM to an "excited" level of, say, 300 nM shifts the voltage gating to about -200 mV. (Although we will save it until another day, the Ca^{2+} level itself is controlled by a series of different Ca^{2+} channels—on different membranes yet—that are in turned controlled by other signal-substances, and Ca^{2+} levels are also set by Ca^{2+} pumps.)

The idea of voltage-gated molecular gates is old. (In 1952, this concept was advanced by Hodgkin and Huxley to explain the differences in conductance that they observed. By the1970's, the terminologies

"potassium channel" and "sodium channel" were widely in use.) However, direct demonstration of channels in biological membranes awaited the development of methods to study channels individually. (This comment is not intended to diminish the contributions of those who made channels and inserted them into artificial membranes or of those who used mathematics to analyze complex recordings, from which they inferred channels.) The break-through came in 1976; the development was an implementation of a "usable" patch clamp.

Overhead: Patch-clamp technique, and voltage-regulation by the ball and chain model

On the left of this overhead, one notes the use of the "old fashioned" (and still useful) method of recording the electrical conductance of biological membranes. E.g., one electrical sensor is inserted into the cell, whereas the second (or reference) is outside the cell. As we discussed earlier, electrical potentials are not absolute numbers, but instead the expressed potential is a difference between two points—in a wall socket, between the "hot" wire and the ground, which is connected to the power station in St. Marks. When an electrogenic transport protein, be it a pump, carrier, or channel, operates in the absence of compensatory events, the membrane potential will be altered. E.g., if the proton pump of the plasmalemma is turned on, the membrane potential will be driven to a more negative value (hyperpolarized, to increase in magnitude). On the other hand, if a potassium inward-rectifying channel opens and potassium passes through it, the membrane potential will be hypopolarized (sent toward zero, in the case of the plasmalemma, made less negative.) As you correctly infer, it is the complexity of the electrical activity on the plasmalemma that prevents one from easily sorting out such-and-such source of activity from another. (In some cases, however, an investigator may stumble on a certain condition that for a window of time will be selective and activate preferentially a single electrogenic activity.) On the overhead, one notes the second method of recording—single cell recording with a patch clamp pipette. In this case, the highly polished small tip of a pipette is appressed on the membrane. A slight suction through the pipette brings it into snug contact, which is the key point, because if the pipette and the membrane were only proximate, electrical conductivity may be effected not through the membrane itself, but also **between** the pipette and the membrane. (Again, the electrical potential to be measured is a comparison of two regions—the outside solution and the interior of the pipette so that electrical conductivity of the membrane is monitored.) Then, in one implementation of the method, the pipette is withdrawn from the cell, which tears off a portion of the membrane that is being held snuggly against the pipette tip. *Viola*—one has a small membrane "patch," in

which there are at most relatively few channels. (If the density of channels is still too high, the channels can be "diluted" by addition of lipids to the biological bilayer.) Then, tests of ligand activation can easily be carried out by altering the solution in the bathing medium or in the pipette. Similarly, voltage gating can be studied by setting at will the membrane potential. At the bottom of the overhead, one sees the resulting electrical recording. In the lower recording, one can see the opening and closing of a single channel. In a membrane patch that contains several channels, one sees the conductance change in steps: the channel state is a binary condition.

Finally, we turn our attention to the regulation of the opening and closing state of a single kind of regulated channel: the voltage-gated channel. As we have discussed so many times before, a membrane protein laces back and forth across the membrane, a potassium channel, about four times. Either termini (COO^- or $NH3^+$) may dangle into whichever aqueous phase, and as we saw for the P-type ATPase, a hydrophilic portion of a loop may extend functionally into the cytosol. There are two current ways to explain the voltage dependence of channels. One way, the so-called ball-and-chain model, as the name implies, involves a globular portion of the protein that is tethered to the membrane-embedded portion. When the channel is closed, the ball has moved into position to block the channel mouth. When the membrane potential changes to a permissible difference, it is supposed that charges on the membrane-embedded portion of the protein are redistributed; these charges repulse the "ball" and the channel is open.