Robert W. Putnam

22

Intracellular pH Regulation

I. Introduction

Intracellular pH is an important aspect of the intracellular environment. Changes in intracellular pH can potentially affect virtually all cellular processes, including metabolism, membrane potential, cell growth, movement of substances across the surface membrane, state of polymerization of the cytoskeleton, and ability to contract in muscle cells. Changes of intracellular pH are also often one of the responses of cells to externally applied agents, including growth factors, hormones, and neurotransmitters. Further, many organelles, such as lysosomes, mitochondria, and endosomal vesicles, maintain an organellar pH that is different from the cytoplasmic pH (pH_i), and these pH differences have important functional consequences for those organelles. It is thus not surprising to find that cells have elaborated a variety of mechanisms that enable them to regulate their intracellular pH. In this chapter, we will discuss the pH level in the cytoplasm and various compartments of a cell, the variety of mechanisms available to a cell to regulate its pH_i, and the functional consequences of changes in pH_i.

II. pH and Buffering Power

The concept of pH was first introduced in 1909 by Sörenson and defined as $-\log[H^+]$. This term was a more convenient way to express the concentration of an ion that is present at very low concentrations. Incorporating the concept of activity, pH is defined as

$$pH = -\log\left(a_H\right) = -\log\left(\gamma_H\left[H^+\right]\right) \tag{1}$$

where a_H is the activity of H^+ and γ_H is the activity coefficient of H^+. At normal intracellular ionic strength, γ_H is about 0.83.

Protons tend to bind to macromolecules, and thus are usually present at very low concentrations in biological solutions. This property is the basis for **buffering power.** A variety of weak acids and bases can bind H^+ through reversible equilibrium binding reactions. Thus, a weak acid in solution obeys the equilibrium reaction

$$HA \rightleftharpoons H^+ + A^- \tag{2}$$

where HA is the weak acid (e.g., lactic acid) and A^- is the conjugate weak base (e.g., lactate). This equilibrium is described by an apparent equilibrium constant, K_a', as

$$K_a' = a_H \cdot \frac{[A^-]}{[HA]} \tag{3}$$

This equation is more familiar in its logarithmically transformed expression,

$$pH = pK_a' \log\frac{[A^-]}{[HA]} \tag{4}$$

where pK_a' is $-\log K_a'$. This equation, better known as the **Henderson-Hasselbalch equation,** describes the thermodynamic equilibrium that holds for a weak acid in a solution of constant pH. The Henderson-Hasselbalch equation is most commonly used in its specialized form for the total reaction of the hydration of CO_2 and the dissociation of the resulting carbonic acid into H^+ and bicarbonate as

$$pH = pK_a' + \log\frac{\left[HCO_3^-\right]}{\alpha \cdot P_{CO_2}} \tag{5}$$

where α is the solubility coefficient of CO_2 in a given solution and P_{CO_2} is the partial pressure of CO_2 in that solution. Two important facts can be deduced from the Henderson-Hasselbalch equation. First, in any weak acid solution, there will be a finite amount of both A^- and HA. For example, if HCO_3^- is added to a solution, CO_2 will be generated and thus be present. Conversely, if CO_2 is bubbled through a solution, HCO_3^- will be produced. Second, this equation can be used to calculate any of the variable parameters if the other three are known. For instance, if a solution is equilibrated with a gas of known P_{CO_2}, the $[HCO_3^-]$ in that solution can be calculated from Eq. 5 once the pH has reached a stable value (values for pK_a' are readily available).

On addition of H^+ (or OH^-) to a solution, the pH will change. However, if the solution contains weak acids (or bases), many of the added protons (or hydroxyl ions) will be bound up, thus minimizing the change in the concentration of free H^+ and thereby minimizing the change in pH. Since these substances minimize the change in pH upon addition of acid or base, weak acids and bases are referred to as *buffers*. The definition of the buffering power (β) of a solution is

$$\beta = \frac{d[B]}{d\text{pH}} \tag{6}$$

where $d[B]$ is the amount of base added to the solution and dpH is the change in pH of the solution due to that base addition. The addition of acid to the solution is equivalent to a negative addition of base, $-d[B]$. The units of β are mM/pH unit.

An example will indicate the importance of buffering power to maintaining the pH of a solution. If 1 mM NaCl is added to a solution, $[Na^+]$ and $[Cl^-]$ increase by 1 mM (for simplicity, the effects of the nonideal activity coefficients will be ignored). However, the addition of 1 mM HCl to a solution that has a pH of 7.0 and a buffering power of 10 mM/pH unit will cause that $[Cl^-]$ to increase by 1 mM, but will cause the pH to decrease by only about 0.28 pH unit, to 6.72. Thus, of the added 1 mM of H^+, only 0.091 μM remain free, that is, only 1 out of every 11 000 added H ions remains free. The rest are bound to the weak acid buffers. If the buffers had not been present, the same addition of 1 mM HCl would have changed the solution pH by about 4 units to pH 3. This clearly demonstrates that the presence of buffers in a solution markedly blunts the effects of added acid or base.

A buffer can act as either a **closed buffer** or an **open buffer.** A closed buffer is one in which the total buffer concentration remains constant. Most of the commonly used laboratory buffers, such as Hepes or Tris, operate as closed buffers in solution. If a buffer is a weak acid ($HA \rightleftharpoons A^- + H^+$), then it operates as a closed buffer when the concentration of the total acid ($[A]_T = [HA] + [A^-]$) remains constant. Such a buffer can become protonated or deprotonated, but the total amount of buffer does not change. The following is a mathematical expression for the buffering power of a weak acid acting as a closed buffer:

$$\beta_{\text{closed}} = \frac{2.303[A]_T K_a' a_H}{\left(K_a' + a_H\right)^2} \tag{7}$$

where K_a' is the apparent dissociation constant of the weak acid. Several conclusions can be derived from this equation. When pH is very high ($a_H \rightarrow 0$) or very low ($a_H \rightarrow \infty$), β_{closed} approaches 0. β_{closed} reaches a maximum when $a_H = K_a'$ (i.e., when pH = pK_a') and $\beta_{\text{closed}}^{\text{max}} = 0.58[A]_T$. The relationship between pH and β_{closed} for a theoretical closed buffer is shown in Fig. 1.

In contrast to the conditions for a closed buffer, if the protonated (or uncharged) form of a buffer remains constant (i.e., [HA] = constant), then the buffer operates as an open buffer. The most common example of an open buffer in solution is CO_2/HCO_3^-. Such a solution contains HCO_3^- and is equilibrated with gaseous CO_2 (usually by bubbling). If acid is added to such a solution, H^+ combines with HCO_3^- and forms additional CO_2. Since the solution is in equilibrium with a

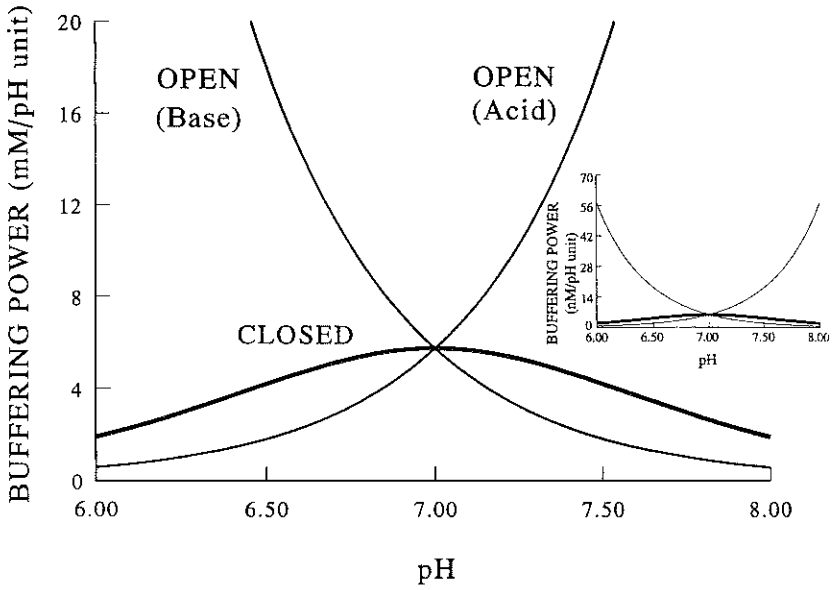

FIGURE 1. The pH dependence of a buffer operating as a closed or open buffer. The buffering powers for both a weak acid buffer ($H^+ + A^- \rightleftharpoons HA$) and weak base buffer ($H^+ + B \rightleftharpoons BH^+$) are plotted. The buffers are assumed to have a pK_a' of 7.0 and to have the same buffering power at pH 7.0 whether operating as an open or closed buffer. Note that while closed buffers have maximal buffering power when pH = pK_a', the buffering power for an open buffer is higher at pH values higher than pK_a' (for weak acid buffers) or at pH values lower than pK_a' (for weak base buffers). Inset: the same plot with a different scale for the ordinate. Note how much higher the open buffering power is compared with the closed buffering power at pH values well above (weak acid) or below (weak base) the value of pK_a'.

fixed P_{CO_2}, the additional CO_2 diffuses from the solution and is removed. Thus, under these conditions, the amount of the uncharged form of the buffer (CO_2) remains constant, whereas the total buffer amount goes down because the $[HCO_3^-]$ has decreased. The buffering power of an open buffer is given by

$$\beta_{open} = 2.303[A^-] \quad (8)$$

where $[A^-] = [HCO_3^-]$ for the open buffering power of CO_2/HCO_3^-. The relationship between pH and β_{open} for a theoretical open buffer is shown in Fig. 1.

Open buffers differ from closed buffers in two important respects. First, unlike closed buffers, the buffering power of open buffers is not maximal at their pK_a' values. In fact, open buffers become better buffers at the more extreme values of pH (Fig. 1). β_{open} becomes larger with alkalinization for weak acids and with acidification for weak bases. Second, open buffers have a much higher buffering power than closed buffers under similar conditions (Fig. 1). Note, however, that neither Eq. 7 nor Eq. 8 includes any indication of the nature of the buffer. Thus, all closed buffers are equally potent when the pH is at their pK_a' and all open buffers are equally potent when they have the same $[A^-]$.

Since most uncharged substances are substantially more permeant through cell membranes than charged species, **almost all weak acids and bases can act as open buffers in cells.** For example, a weak acid that has a pK_a' of 4.0 acts like a closed buffer in solution, and since its pK_a' is 4.0, it would be a poor buffer at pH 7.0. However, inside a cell at pH 7.0, this weak acid acts like an open buffer. Any added H^+ will bind to the anionic form of the buffer. The uncharged buffer molecule formed will readily diffuse from the cell and be removed by the blood. Thus, although this weak acid is a poor buffer in the medium, it can contribute substantially to the buffering power inside a cell if present at sufficient concentration.

Complex solutions, like blood, contain multiple buffers. The **total buffering power** (β_{total}) of such solutions will be the sum of the various buffers; that is, buffers operate independently in solution. Thus,

$$\beta_{total} = \sum \beta_{closed} + \sum \beta_{open} \quad (9)$$

Finally, it is inherent in the definition of buffering power (Eq. 6) that buffering power is a coefficient that can be used to convert a change of pH into a change in the amount of proton equivalents moved. This relationship has practical application. For instance, in the study of the regulation of intracellular pH, the activity of a transporter that moves H^+ across the surface membrane (such as the Na^+-H^+ exchanger, see Section VI) is determined by measuring the rate of change of pH_i (dpH_i/dt). The movement of H^+ on this transporter is accompanied by Na^+, whose movement is measured as a radioisotopic flux (amount of Na^+ influx per unit time). To compare the flux of Na^+ with the flux of H^+, the rate of change of pH_i needs to be converted to the amount of H^+ moved per unit time. This is accomplished by the following equation

$$J_H = \frac{dpH}{dt} \cdot \beta_{total} \quad (10)$$

where J_H is the flux of protons and has units of mM H^+ per unit time. Using this equation, the flux of H^+, calculated from the measured rate of pH change, can be directly compared to the flux of another ion determined with radioisotopes.

III. Intracellular pH

Protons are just like any other cation, except for three distinguishing characteristics: (1) H ions are a dissociation product of water molecules ($H_2O \rightleftharpoons H^+ + OH^-$) and thus are always present in aqueous solutions, (2) H ions are present at very low concentrations in most solutions, and (3) H ions have much higher mobility than other cations. However, the equilibrium distribution and movement of H^+ across biological membranes are governed by the same principles that govern the movement of all other ions across biological membranes.

Originally, protons were assumed to be at equilibrium across biological membranes because of their very high mobility. Assuming an extracellular pH (pH_o) of 7.4, a V_m of –60 mV (inside negative), and assuming that H ions are passively distributed across the membrane (i.e., at equilibrium), pH_i would be 6.4 (calculated from the Nernst equation). However, at such an intracellular pH, metabolism and a variety of other cellular functions would be impaired. With the advent of modern reliable techniques for measuring intracellular pH, including **pH-sensitive glass microelectrodes** and **pH-sensitive fluorescent dyes** (see Appendix), it was shown that in the majority of cells pH_i was between 6.8 and 7.2, well above the calculated value for equilibrium pH. It is now clear that for most cells (with the notable exception of red blood cells), pH_i is considerably more alkaline than it would be if protons were at passive equilibrium across the cell membrane.

The question still remains how pH_i can be well above the equilibrium value, since H^+ should be highly permeant to most cell membranes. In fact, the **permeability of H^+** across biological membranes has been estimated to be between 10^{-4} and 10^{-2} cm/s, about four orders of magnitude higher than typical K^+ permeabilities. However, it is the conductance, and not the permeability, of H^+ that is crucial. **Conductance** is a measure of ion flux and is a function of both the permeability and the free concentration of an ion. H ions have low conductance across biological membranes despite their high permeability, because they are present in such low concentrations (10^{-7} M free concentration for H^+ versus 10^{-1} M free concentration for K^+). Thus, the acidifying influx of H ions (down their electrochemical gradient) will be small, and these H ions can easily be removed from the cell by membrane transport systems (see Section VI).

In summary, most cells have a cytoplasmic pH that is more alkaline than the value calculated assuming equilibrium of H^+ across the cell membrane, and pH_i for most cells is about 6.8–7.2.

IV. Organellar pH

Several intracellular organelles independently control their internal pH, which differs from the cytoplasmic pH (Fig. 2). These organelles include **mitochondria** and **acidic intracellular organelles.**

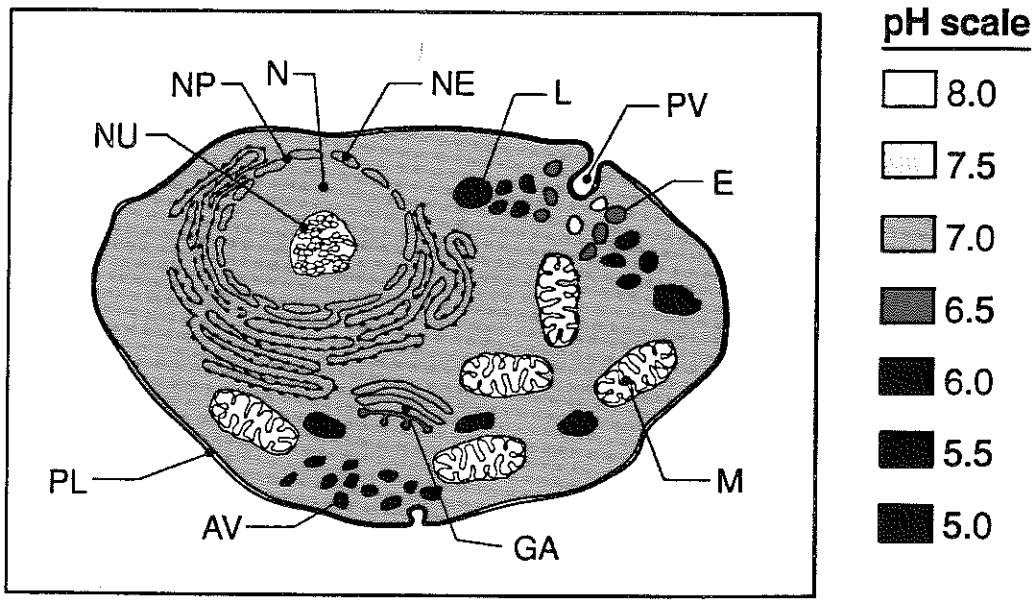

FIGURE 2. A diagram of an idealized cell with the gray scale representing different values of pH (note calibration scale on the right). The cytoplasm has a fairly uniform pH of about 7.0, but note the alkaline mitochondria, the increasing acidification in the Golgi apparatus and in the endosomes, and the very acidic lysosomes. AV—acidic vesicles; E—endosomes; GA—Golgi apparatus; L—lysosomes; M—mitochondria; N—nucleus; NE—nuclear envelope; NP—nuclear pore; NU—nucleolus; PL—plasmalemma; PV—pinocytotic vesicle.

A. Mitochondria

One of the major roles of mitochondria is the production of ATP. A proton gradient across the inner mitochondrial membrane is required for the production of ATP. The electron transport chain translocates protons from the mitochondrion to the cytoplasm across the inner mitochondrial membrane. This proton extrusion creates an electrical and chemical gradient for proton influx into the mitochondrion. This H^+ influx occurs through a membrane-bound ATPase that produces ATP upon passive proton flux back into the mitochondrion. This is known as the **chemiosmotic hypothesis.**

The extrusion of H^+ to establish a proton gradient renders mitochondria alkaline relative to the cytoplasm by about 0.3–0.5 pH unit. Thus, intramitochondrial pH can be between 7.5 and 8.0. In addition, this pH gradient is maintained in the face of considerable acid loads, indicating that mitochondria may well be able to regulate their internal pH independently of cytoplasmic pH.

B. Acidic Intracellular Organelles

Organelles with a markedly acidic interior are those involved in either the endocytic pathway or the secretory pathway. The acidic organelles involved in endocytosis include **coated pits, endosomes** (i.e., prelysosomal endocytic vesicles), and **lysosomes.** Acidic vesicles in the secretory pathway include the **Golgi apparatus** (or at least part of it) and **storage granules** for amines (e.g., chromaffin granules involved in catecholamine secretion) and peptides (e.g. secretory granules in the endocrine pancreas). The pH is not known for all of these compartments, but can be as low as 4.5–5.0 in lysosomes and 5.0–5.7 in endosomes and secretory granules. The pH in the Golgi apparatus seems to fall the farther the compartment is from the nucleus, with values of 6.2–6.4 in the medial Golgi and values as low as 5.9 in the trans Golgi network (TGN).

The acidic internal environment of these various organelles is believed to be necessary for their function. For example, the primary function of lysosomes is the biochemical degradation of macromolecules, and these organelles contain a large number of hydrolytic enzymes whose pH optima are about pH 5.0. This serves as protection for the cell. If the lysosomes should leak, the hydrolytic enzymes would be inactivated by the high cytoplasmic pH, thus preventing the indiscriminate degradation of important macromolecules. Receptor-mediated endocytosis involves the internalization of ligand-receptor complexes in endocytic vesicles, or endosomes. Acidification of these vesicles is essential for the dissociation of the ligand from the receptor within the endosome. Once the ligand has dissociated, the internalized receptor is recycled to the surface membrane, while the ligand is delivered to the lysosome. Finally, secretory granules (and perhaps part of the Golgi) serve to accumulate macromolecules to be secreted, and often mediate processing or modification of these substances. The maintenance of an acidic environment in these granules can be crucial to both of these functions. There is evidence that the large outward H^+ gradient is used to accumulate biogenic amines in amine secretory granules. This accumulation may be mediated by an H^+-amine exchanger in the granule membrane. These accumulated substances can be biochemically modified into their final form, and the enzymes responsible for these modifications often have low pH optima or depend on the availability of organic compounds that will accumulate only in

acidic compartments. For all of these acidic compartments, then, it is clear that their proper functioning depends on the maintenance of a low internal pH.

C. Nucleus

The nucleus is separated from the cytoplasm by a double-membrane system that has large nuclear pores. Large macromolecules (up to 5 kDa) readily permeate the nuclear pores and rapidly come to equilibrium between the nucleus and cytoplasm. Given this high degree of permeability of the nuclear membrane, it was believed that nuclear pH could not differ much from cytoplasmic pH. However, recent measurements clearly show that nuclear pH is from 0.1 to 0.5 pH unit more alkaline than cytoplasmic pH. These data imply that the nuclear pores do indeed represent a barrier to the free movement of H ions between the nucleus and cytoplasm.

V. Maintenance of a Steady-State pH_i

If a cell is maintaining a **steady-state pH_i** (i.e., the pH of the cytoplasm), the rate of acid loading must be equal to the rate of acid extrusion from the cell (in these terms it is exactly equivalent if an acid molecule moves in one direction or a base molecule moves in the opposite direction) (Fig. 3). Several processes can contribute to acid loading of a cell, including metabolic production of acid, passive influx of H^+ across the cell membrane, leakage of H^+ from acidic internal compartments, pumping of H^+ from alkaline internal compartments, and active influx of H^+ or active extrusion of base. Conversely, acid extrusion includes metabolic consumption of H^+, sequestration of H^+ in internal compartments, and active extrusion of acid or active influx of base.

A. Metabolic Production and Consumption of Acids

Several metabolic reactions involve the production of H^+ (Table 1). These processes include the production of CO_2, glycolysis (through the generation of lactic and pyruvic acids), formation of creatine phosphate, ATP hydrolysis, lipolysis, triglyceride hydrolysis, the generation of superoxide, and the operation of the hexose monophosphate shunt. Obviously, when these reactions run in the opposite direction (e.g., creatine phosphate hydrolysis, ATP formation, or consumption of CO_2), there is a net consumption of H^+ and the cell will alkalinize.

When the cell is at steady state, these reactions must be balanced, and thus the levels of cellular metabolites such as ATP, creatine phosphate, CO_2, and lactate will also be at steady state. However, during transient periods, these metabolic reactions

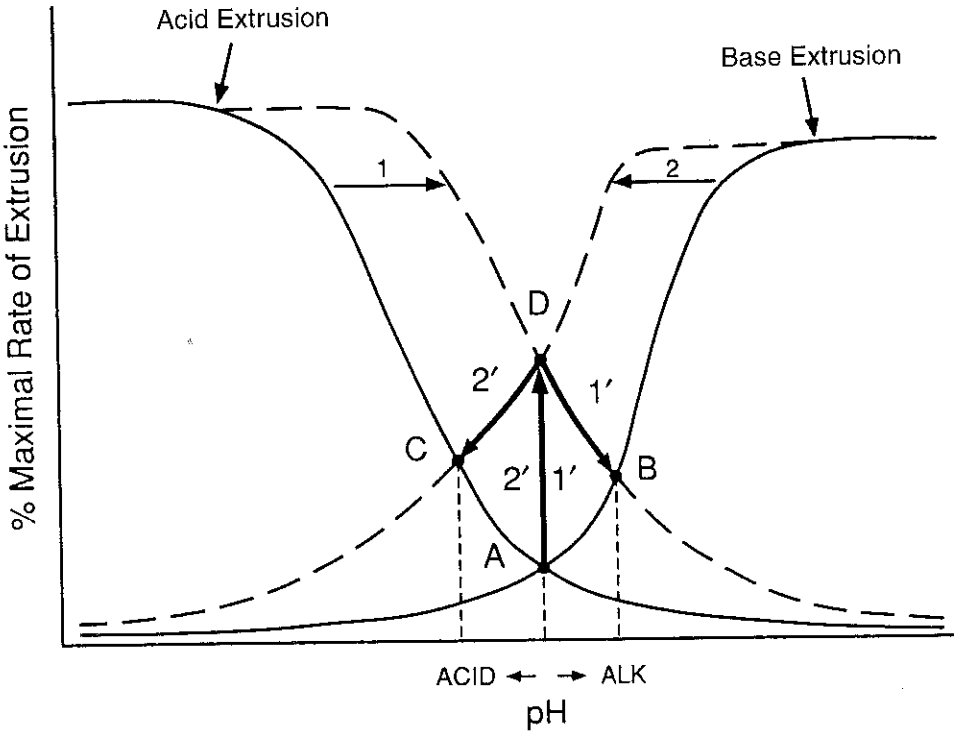

FIGURE 3. A model for the regulation of steady-state pH_i. In the cell, steady-state pH_i is determined as the point where acid-extruding processes are balanced by base-extruding (or acid influx) pathways (point A). If acid-extruding processes are activated (arrow 1—represented as a shift to the right of the pH versus % maximal rate curve), as occurs when growth factors activate Na^+-H^+ exchange, the rate of acid extrusion exceeds the rate of base extrusion and the cell alkalinizes (arrows 1′). Eventually, the cell alkalinizes to the point where acid and base extrusion are again equal and a new alkaline steady state pH_i is reached (point B). In contrast, if base extrusion is activated (arrow 2—represented as a shift to the left of the pH versus % maximal rate curve), as occurs with an increase in the activity of Cl^--HCO_3^- exchange, the rate of base extrusion exceeds acid extrusion and the cell will acidify (arrows 2′). Eventually, the cell acidifies to the point where acid and base extrusion are again equal and a new acidic steady-state pH_i is reached (point C). If acid and base extrusion are activated to the same extent, the steady state pH_i will remain unchanged (point D).

TABLE 1 Examples of Various Metabolic Reactions That Involve the Generation or Consumption of H^+

Glycolysis[a]	Glucose + $2MgADP^-$ + $2P_i^{2-}$ → 2(lactate)$^-$ + $2MgATP^{2-}$ $2MgATP^{2-}$ → $2MgADP^-$ + $2P_i^{2-}$ + $2H^+$
Glycogenolysis[b]	Glycogen + $3P_i^{2-}$ + $3MgADP^-$ + H^+ → $3MgATP^{2-}$ + 2(lactate)$^-$ + glycogen
Creatine phosphate hydrolysis	H^+ + creatine phosphate^{2-} + $MgADP^-$ → $MgATP^{2-}$ + creatine
Lipolysis	Triglyceride → 3(palmitate)$^-$ + $3H^+$ 3(palmitate)$^-$ + $3MgATP^{2-}$ + $3CoA^{4-}$ → 3(palmitoyl CoA)$^{4-}$ + $3AMP^{2-}$ + $6P_i^{2-}$ + $3H^+$ + $3Mg^{2+}$
Superoxide formation	$NADPH + 2O_2 \rightarrow 2O_2^- + NADP^+ + H^+$
Hexose-monophosphate shunt	$G6P^- + 12NADP^+ + 6H_2O \rightarrow NADPH + P_i^- + 12H^+ + 6CO_2$

[a] At pH 7.2 and high $[Mg^{2+}]$, $2H^+$ are always generated but the balance of H^+ produced by the two reactions varies with pH and Mg.

[b] Net H^+ production when hydrolysis of the 3MgATP is taken into account.

can result in net changes in metabolite concentrations and thus contribute markedly to changes in pH_i. For instance, during periods of ischemia, cellular lactate and CO_2 may accumulate while ATP will be hydrolyzed. All of these reactions can contribute to the observed cellular acidification. The hydrolysis of creatine phosphate during ischemia consumes H^+ and will blunt the cellular acidification. Another example of a transient effect of metabolism on cellular pH is the initial acidification seen upon activation of neutrophils with phorbol esters. In this case, stimulation of the production of superoxide and activation of the hexose monophosphate shunt results in increased production of H^+.

B. Passive Transmembrane Flux of H^+

Although the H^+ permeability of most biological membranes is quite high ($P_H \approx 10^{-3}$ cm/s), the actual H^+ flux across the membrane is quite low because of the low free H^+ concentration. For example, the putative H^+ flux across a frog muscle fiber can be calculated. Assuming a constant electric field across the membrane, $P_H = 10^{-3}$ cm/s, $V_m = -90$ mV, $pH_i = 7.2$, $pH_o = 7.35$, and $\beta_{total} = 26$ mM, passive H^+ influx would result in a cellular acidification of only 0.02 pH unit/hour. Although negligible, this does represent a continued acid load on the cell, and if mechanisms do not exist to remove these H ions, the cell will eventually acidify toward the equilibrium pH_i value.

H^+ currents associated with **proton channels** have been reported in several cells, including snail neurons, salamander oocytes, and a number of mammalian cells. All of these channels are activated by depolarization and the resulting H^+ currents alkalinize the cell. Other characteristics of these channels include inhibition by divalents (such as Zn^{2+} and Cd^{2+}), gating by both intracellular and extracellular pH, and low single-channel conductance. These channels have also been shown to have a very high temperature dependence, an unusual feature for an ion channel. It has recently been proposed that these channels can be classified into four isoforms based on channel kinetic properties. These various isoforms include *n* channels (in snail neuron), *o* channels (in oocyte), *e* (for epithelial) channels (in rat alveolar epithelium and frog proximal tubule), and *p* (for phagocyte) channels (in macrophage, neutrophil, CHO, microglia, and myotube, among others). The significance of these channels is not known, but they may contribute to the maintenance of pH in a restricted submembrane space within the cell or contribute to pH_i regulation in cells undergoing prolonged depolarization, such as the prolonged depolarizing fertilization potential in oocytes.

C. Internal Compartments

If the pH values of intracellular compartments, such as mitochondria and lysosomes, are at steady state, then they should have no impact on cytoplasmic pH. However, under pathological conditions where mitochondria or acidic intracellular compartments are rendered leaky or mitochondria take up cytoplasmic Ca^{2+} in exchange for H^+, these compartments could influence pH_i. Under normal conditions, though, these compartments should contribute little to the maintenance of a steady-state pH_i, especially owing to their relatively small volume compared with total cell volume.

It is clear that, at the very least, cells face a continuous acid load from passive H^+ influx. In addition, under many conditions of metabolic stress, cells also experience a metabolic acid load. Thus, cells must possess active extrusion mechanisms to maintain a steady-state pH_i well above the equilibrium value for pH_i.

VI. Active Membrane Transport of Acids and Bases

Several integral proteins within the surface membrane of cells are specialized for the active transport of acids and bases across the membrane. Because of their importance to cellular pH regulation, these transport pathways have been extensively studied and can be divided into five classes: (1) those that move H^+ directly in exchange for another cation; (2) those that move HCO_3^-, or an associated species like CO_3^{2-}; (3) H^+-ATPases (proton pumps) that use energy from ATP hydrolysis to transport H^+; (4) those that cotransport anionic weak bases with Na^+; and (5) those that transport anionic weak bases in exchange for Cl^-.

A. Cation-H^+ Exchangers

The best characterized of the cation-H^+ exchangers is the **Na^+-H^+ exchanger (NHE)** (model 1 in Fig. 4). This exchanger responds to cellular acidification by extruding one H^+ in exchange for the influx of one Na^+ (1:1 stoichiometry means that the exchanger is **electroneutral,** i.e., it does not involve net charge movement). There are now known to be at least five isoforms of the Na^+-H^+ exchanger in mammalian cells. NHE-1 is found in virtually all cells, is inhibited by the loop diuretic

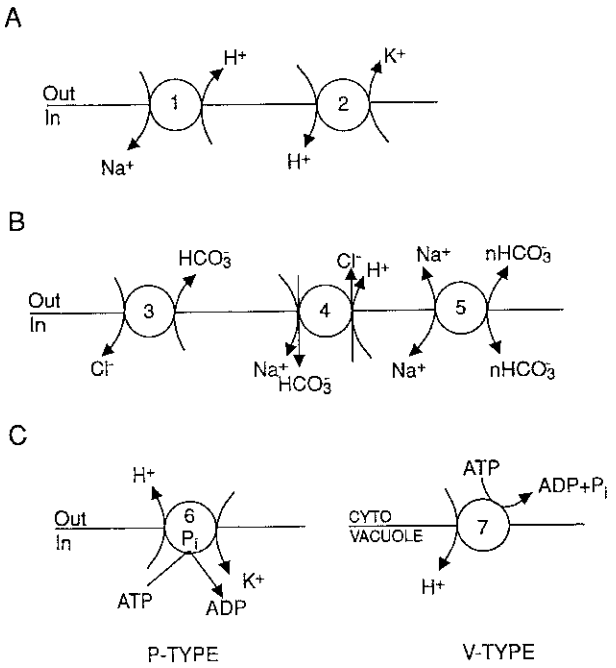

FIGURE 4. Models of several different types of pH-regulating transporters. (A) Cation-H^+ exchangers. Included in this group are the alkalinizing Na^+-H^+ exchanger (1) and the acidifying K^+-H^+ exchanger (2). (B) HCO_3^--dependent transporters. This group includes the Cl^--HCO_3^- exchanger (band 3 from red blood cells) (3). Another transporter in this group is the (Na^++HCO_3^-)-Cl^- exchanger (4). Shown here is merely one possible model for this exchanger. This transporter could involve the influx of two HCO_3^- instead of the influx of one HCO_3^- and the efflux of an H^+. Alternatively, one CO_3^{-2} or two $NaCO_3^-$ could be transported in. These four different variants have the same effect on pH, but can be partially distinguished kinetically. The Na^+-HCO_3^- cotransporter is shown as model 5. This transporter is electrogenic, mediating the movement of n HCO_3^- for each Na^+, and is thus sensitive to membrane potential (V_m). The direction of cotransport depends on the value of V_m. (C) Two different types of H^+-ATPases. The P-type H^+-ATPase (6), typified by the electroneutral gastric H^+,K^+-ATPase, involves a phosphorylated intermediate. The V-type H^+-ATPase (7), typified by the vacuolar H^+-ATPase, does not produce a phosphorylated intermediate.

amiloride and its analogs, can be activated by a variety of agents, and has a molecular weight of 91 000. NHE-2, which has only 50% amino acid homology with NHE-1, is found predominantly in the gastrointestinal tract (GI tract) and kidney, is much less sensitive to inhibition by amiloride analogs than NHE-1, and has a molecular weight of about 91 000. NHE-3 has about 40% amino acid homology with NHE-1, has a molecular weight of 93 000, is expressed largely in the GI tract and kidney, and is not readily inhibited by amiloride. NHE-4 also has about 40% amino acid homology with NHE-1, is the smallest NHE isoform with a molecular weight of 81 000, is largely expressed in the GI tract (with some expression in the uterus, brain, and kidney), and is not very sensitive to inhibition by amiloride. NHE-5 has recently been characterized from humans and rats. This isoform is most similar to NHE-3 (62% amino acid homology), but has a larger molecular weight, 99 000, than any of the the other NHE isoforms. This isoform is not very sensitive to inhibition by amiloride analogs. NHE-5 is largely expressed in the brain of rats, especially in neuronal cell bodies. Other NHE isoforms undoubtedly exist in mammalian cells. A sixth isoform, NHE-6, has been described in human mitochondria. In addition, a Cl-dependent Na^+-H^+ exchanger, described in colonic crypt cells, might represent yet another mammalian NHE isoform. Other NHE isoforms also exist in cells from lower vertebrates, invertebrates, plants, and bacteria. For instance, an unusual isoform, β-NHE, that is 50–75% homologous to NHE-1 and is activated by cAMP (unlike NHE-1) has been described in trout red blood cells. The significance of these different isoforms is still largely unclear, but given the variety of functions performed by NHE in cells, it is not surprising that multiple forms have arisen.

The basic structure of the five mammalian plasma membrane NHE isoforms is similar (Fig. 5A), containing two major domains, a transmembrane domain and a cytoplasmic domain.

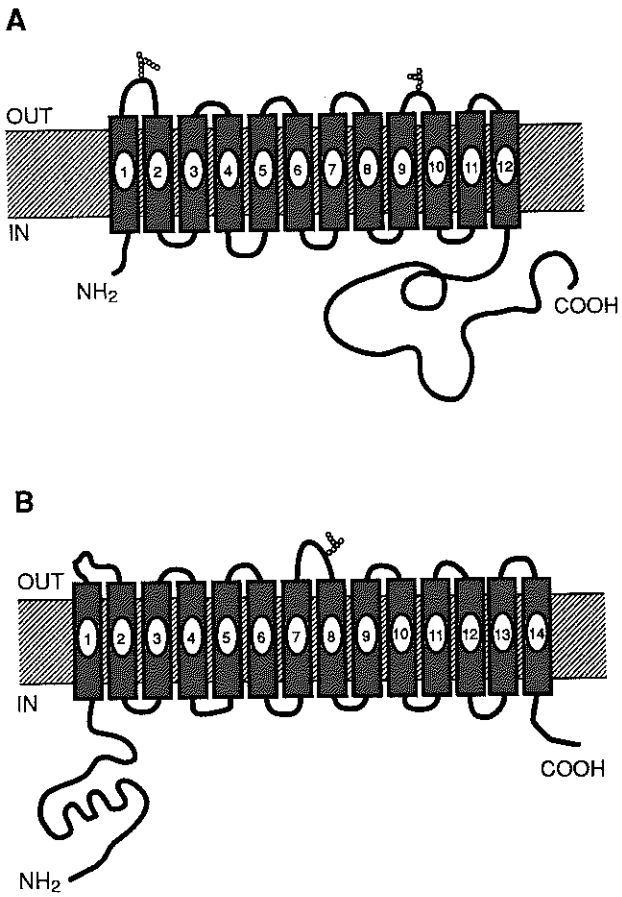

FIGURE 5. Models of (A) the structure of the Na^+-H^+ exchanger (NHE-1) and (B) the Cl^--HCO_3^- exchanger (AE-1). The Na^+-H^+ exchanger is believed to have 12 membrane-spanning regions with a large cytoplasmic domain at the C-terminal end. This cytoplasmic domain contains several potential phosphorylation sites that are important to the regulation of the Na^+-H^+ exchanger. The N-terminal end is also cytoplasmic. Two potential glycosylation sites are shown. The Cl^--HCO_3^- exchanger is believed to have 14 membrane-spanning regions, and both terminal ends are cytoplasmic. The large cytoplasmic domain is on the N-terminal end in the Cl^--HCO_3^- exchanger. This cytoplasmic domain (in red blood cells at least) contains many binding sites, including those for the cytoskeletal element ankyrin, for hemoglobin, and for some glycolytic enzymes.

The transmembrane domain (N-terminal ~500 amino acids) is believed to have 12 membrane-spanning regions with an N-linked glycosylation site on the first external loop. This means that NHE is a glycoprotein. The cytoplasmic domain (C-terminal ~300 amino acids) represents a large cytoplasmic region with available serine phosphorylation sites. Both the N- and C-terminal ends of NHE are cytoplasmic. It is believed that the cytoplasmic C-terminal end is involved in the regulation of NHE while the membrane-spanning regions are responsible for Na^+ and H^+ transport. This is borne out by the expression of NHE mutants that lack the C-terminal end in fibroblasts that do not have NHE. In these cells, Na^+-H^+ exchange activity is seen, but can no longer be activated by growth factors.

A wide variety of substances have been shown to activate NHE, including hormones, neurotransmitters, growth factors, and the extracellular matrix. Many of these factors are believed to activate the NHE by phosphorylating serine residues on the C-terminal end. These factors may also affect the NHE through the binding of a regulatory protein with the exchanger and/or by binding of a Ca^{2+}-calmodulin complex to a putative autoinhibitory domain on the exchanger. Activation often involves an increase in the affinity of the exchanger's internal binding site for cytoplasmic H^+ (see arrow 1, Fig. 3). In some cases, activation of NHE may also involve interaction between the exchanger and the cell cytoskeleton. There is accumulating evidence that NHE isoforms may function best as homodimers. The functional significance of dimerization is unclear. Cell acidification also dramatically activates NHE. This activation is due to an internal allosteric H^+ binding site on NHE (distinct from the H^+ transport site) that increases exchange when occupied. Finally, decreased cell volume (cell shrinkage) also activates NHE. This activation does not involve phosphorylation of NHE and appears to be mediated by a unique activation pathway.

Many signaling pathways can activate NHE, depending on the stimulus. For example, growth factor activation of NHE is usually mediated by an elevation of intracellular Ca^{2+} and/or by an increased activity of protein kinase C. These pathways commonly involve phosphoinositide breakdown. Activation of NHE by cell shrinkage appears to involve ATP- and GTP-dependent pathways.

Much progress has been made recently to correlate NHE function with structure. As stated above, the transmembrane domain contains the Na^+ and H^+ transport sites. In addition, this domain contains the external amiloride binding site and the internal H^+ allosteric binding site, although the pK for this latter site is markedly acid in the absence of the C-terminal cytoplasmic domain. The C-terminal cytoplasmic domain contains several putative phosphorylation sites and is involved in the regulation of NHE. This region also contains the autoinhibitory domain, the calmodulin binding site, and a possible binding site for the putative cytoplasmic regulatory factor. The C-terminal domain may also contain sites able to interact with cytoskeletal proteins.

Transgenic mice now exist that lack NHE-1, NHE-2 and NHE-3. All 3 types of mice are viable, but mice lacking NHE-1 have slowed postnatal development and by 14 days exhibit neurologic symptoms (primarily gait problems and seizures). These mice usually die before weaning. Mice lacking NHE-2 are apparently normal, but show long-term gastric changes, including a small decrease in acid secretion and degeneration of parietal cells. Mice lacking NHE-3 also have long-term viability but exhibit gastrointestinal (GI) and renal defects. These defects cause diarrhea, mild acidosis, and lowered blood pressure. These symptoms are consistent with a role for NHE-3 in GI and renal HCO_3^- and fluid absorption. The use of transgenic mice should be helpful in further defining the role of various NHE isoforms.

The other major cation-H^+ exchanger is the **K^+-H^+ exchanger** (model 2 in Fig. 4A). This transporter exchanges intracellular K^+ for extracellular H^+ and results in cellular acidification. K^+-H^+ exchange has been found in nucleated red blood cells and mediates solute efflux during regulatory volume decrease (see Section VII.H). It has also been observed in retinal pigment epithelial cells, where it enables the cell to regulate pH_i in the face of an alkaline load.

B. HCO_3^--Dependent Transporters

These transporters are actually a superfamily of related transport proteins that affect pH_i and are characterized by their ability to transport HCO_3^- (or a related species like CO_3^{2-}) and by the ability of disulfonic stilbene derivatives to inhibit them. The three major types of HCO_3^--dependent transporters are Cl^--HCO_3^- exchange (band 3 from red blood cells, termed AE for anion exchanger), (Na^++HCO_3^-)-Cl^- exchange (often termed Na^+-dependent or Na^+-driven Cl^--HCO_3^- exchange), and electrogenic Na^+-HCO_3^- cotransport (termed NBC for Na^+ bicarbonate cotransport).

The operation of **Cl^--HCO_3^- exchange (AE)** (model 3 in Fig. 4B) has been studied most extensively in red blood cells. This electroneutral transporter involves a 1:1 exchange of Cl^- for HCO_3^- (although many other ions, such as SO_4^{2-}, can also be transported under specific conditions). As with the Na^+-H^+ exchanger, the Cl^--HCO_3^- exchanger has several isoforms, denoted AE 1, 2, and 3. These various isoforms have about 80–90% homology. AE 1 is the band 3 transporter from red blood cells and is the smallest isoform (M_r = 115 000). This protein has as many as 14 membrane-spanning domains (helices) and a large N-terminal cytoplasmic domain that contains various binding sites, including one for ankyrin (Fig. 5B). AE 2 and 3 are larger (M_r = 145 000–165 000). AE 2 appears to be the housekeeping anion exchanger and is widely distributed. It is activated by cellular alkalinization and returns pH_i to normal by extruding base (HCO_3^-) and thereby reacidifying the cell. This exchanger may also possess an internal allosteric regulatory site that activates the exchanger at **alkaline** values of pH_i (see the base extrusion curve in Fig. 3). In addition to its role in the regulation of pH_i, Cl^--HCO_3^- exchange may also play a role in regulating intracellular Cl^-. The function of AE 3 is not yet clear but it has a far more restricted distribution than AE 2, being found only in the heart and the central nervous system.

The **(Na^++HCO_3^-)-Cl^- exchanger** (model 4 in Fig. 4B) was originally described as the pH-regulating transport system in invertebrate nerve and muscle preparations, but has since been found in a wide variety of cells. Because it transports Na^+, it op-

erates in the opposite way than Na^+-independent Cl^--HCO_3^- exchange (i.e., the anion exchangers described above). Thus, the (Na^++HCO_3^-)-Cl^- exchanger exchanges one external Na^+ for one internal Cl^- and neutralizes the equivalent of two internal protons. As such, this exchanger is electroneutral and mediates the alkalinization of the cell in response to an acid load. The ability of this exchanger to neutralize two acid equivalents could be achieved by the influx of two HCO_3^-, the influx of one HCO_3^- in exchange for the efflux of one H^+, the influx of one CO_3^{2-}, or the influx of an ion pair ($NaCO_3^-$). In squid axon, this exchanger has been suggested to involve $NaCO_3^-$-Cl^- exchange, whereas in barnacle muscle fibers it must be another variant, suggesting that this exchanger also has at least two isoforms. The structure of this exchanger is not currently known.

The **Na^+-HCO_3^- cotransporter (NBC)** (model 5 in Fig. 4B) was originally identified in renal epithelial cells but has since been found in a number of other cell types as well. It mediates the movement of one Na^+ with one, two, or three HCO_3^-, depending on the cell type in which the transporter is located. This transporter differs from the other HCO_3^--dependent transporters in that it does not require Cl^- and, in the case where the stoichiometry is 1:2 or 1:3, it is electrogenic. In proximal tubule epithelial cells, NBC is involved in HCO_3^- reabsorption by mediating HCO_3^- efflux across the basolateral membrane. In other cells, the cotransporter is proposed to mediate HCO_3^- influx and contribute to the regulation of intracellular pH in the face of an acid load. The renal cotransporter (1:3 stoichiometry) has been suggested to involve the cotransport of one Na^+ with one HCO_3^- and one CO_3^{2-}.

The NBC and several of its variants have recently been cloned. The first mammalian NBC to be cloned is from rat kidney (rkNBC). This is a glycoprotein consisting of 1035 amino acids (M_r ~130 000). Its structure is similar to other membrane transport proteins, containing at least 10 membrane-spanning regions, a large extracellular loop between membrane-spanning segments 5 and 6, and large cytoplasmic domains (one each at the N- and C-terminal ends). These cytoplasmic domains are probably the site of cotransport regulation and contain putative phosphorylation sites for protein kinases A and C, casein kinase II, and tyrosine kinase. A human kidney clone (hkNBC), with 97% homology to rkNBC, has recently been described. These kidney NBCs are electrogenic, with a stoichiometry of one Na^+ transported for every three HCO_3^- ions.

An NBC from human heart (hhNBC) has recently been described. This clone (along with an identical one from human pancreas) is identical to rkNBC, except that hhNBC has a longer N-terminus (by 44 amino acids); hhNBC is also electrogenic, but with a stoichiometry of 1:2. A similar clone has been isolated from rat brain (rbNBC). This clone has longer C- and N-terminal ends, a stoichiometry of 1:2, and is predominantly localized to cortical neurons but not astrocytes. Finally, a novel clone has been isolated from vascular smooth muscle cells (also found in testis and spleen cells). This clone (NBC_N-1) has a very large N-terminus, is **electroneutral** (stoichiometry of 1:1), and is apparently not inhibited by DIDS.

These NBC clones are about 30–35% identical to AE transport proteins, indicating that these transporters are indeed part of a superfamily. It will be of interest to see the homology of the (Na^++HCO_3^-)-Cl^- exchanger with NBC and AE, once the former has been cloned.

C. H^+-ATPases (Proton Pumps)

There are at least three known varieties of **H^+-ATPases:** (1) F_0-F_1-type ATPase; (2) E_1-E_2 or P-type ATPase; and (3) vacuolar or V-type ATPase. The **F_0-F_1-type ATPase** is found in mitochondria (see Chapter 8). This ATPase has also been called the ATP synthase, since it functions to produce ATP when H^+ moves down its electrochemical gradient. F_0-F_1-type ATPase has a lollipop shape and a membrane-spanning region, F_0, that forms the putative H^+ pore through the membrane as well as an extrinsic head region, F_1, that contains the ATPase activity. The F_0 subunit is quite large, with 5 subunits and a molecular weight of about 380 000. The F_1 region has 4 subunits and a molecular weight of about 100 000. These two regions are connected by a stalk that contains several subunits, one of which confers sensitivity to oligomycin. The F_0-F_1-type ATPase can be inhibited by azide and N,N'-dicyclohexylcarbodiimide (DCCD) in addition to oligomycin.

The **P-type ATPases** (model 6 in Fig. 4C) are characterized by forming a phosphorylated intermediate upon ATP hydrolysis. The classic example of such an ATPase is the Na^+,K^+-ATPase. An example of a **P-type H^+-ATPase** is the H^+,K^+-ATPase, best characterized from the apical membrane of gastric glands, where it is responsible for acid secretion into the stomach. This ATPase has also been implicated in the acidification of the urine and reabsorption of K^+ by the kidney and in the establishment of an H^+ gradient across yeast plasma membrane. In contrast to the F_0-F_1-ATPase, the H^+,K^+-ATPase exchanges one K^+ for one H^+ and is thus electroneutral. The H^+,K^+-ATPase has a molecular weight of about 110 000 and has a structure similar to those of other membrane-bound ATPases with several membrane-spanning regions and a large cytoplasmic domain containing the ATP hydrolysis site. Inhibition by vanadate is characteristic for P-type ATPases.

The third type of H^+-ATPase is the vacuolar, or V-type, ATPase (model 7 in Fig. 4C) which is found in yeast and plant vacuoles as well as in several eukaryotic cells (e.g., kidney cells, osteoclasts, and macrophages) and organelles (e.g., endosomes, lysosomes, secretory granules, and Golgi apparatuses). **V-type ATPases** are more like the F_0-F_1-type ATPase than the P-type ATPase in that they do not form phosphorylated intermediates, are quite large (>400 kDa), assume a lollipop shape, and are electrogenic. The major function of V-type ATPases is the acidification of intracellular organelles (see Section IV.B), which is important for proper protein targeting and handling. These ATPases are characterized by their lack of sensitivity to vanadate and oligomycin and by their inhibition by N-ethylmaleimide (NEM), DCCD, and bafilomycin. While P- and F_0-F_1-type ATPases are found in both prokaryotes and eukaryotes, the V-type ATPases are only found in eukaryotes and therefore presumably evolved more recently.

D. Na^+-Organic Anion Cotransport

In a variety of organisms, renal proximal tubule cells have been shown to have **Na^+-organic anion cotransporters** (model 1 in Fig. 6). These cotransporters mediate the influx

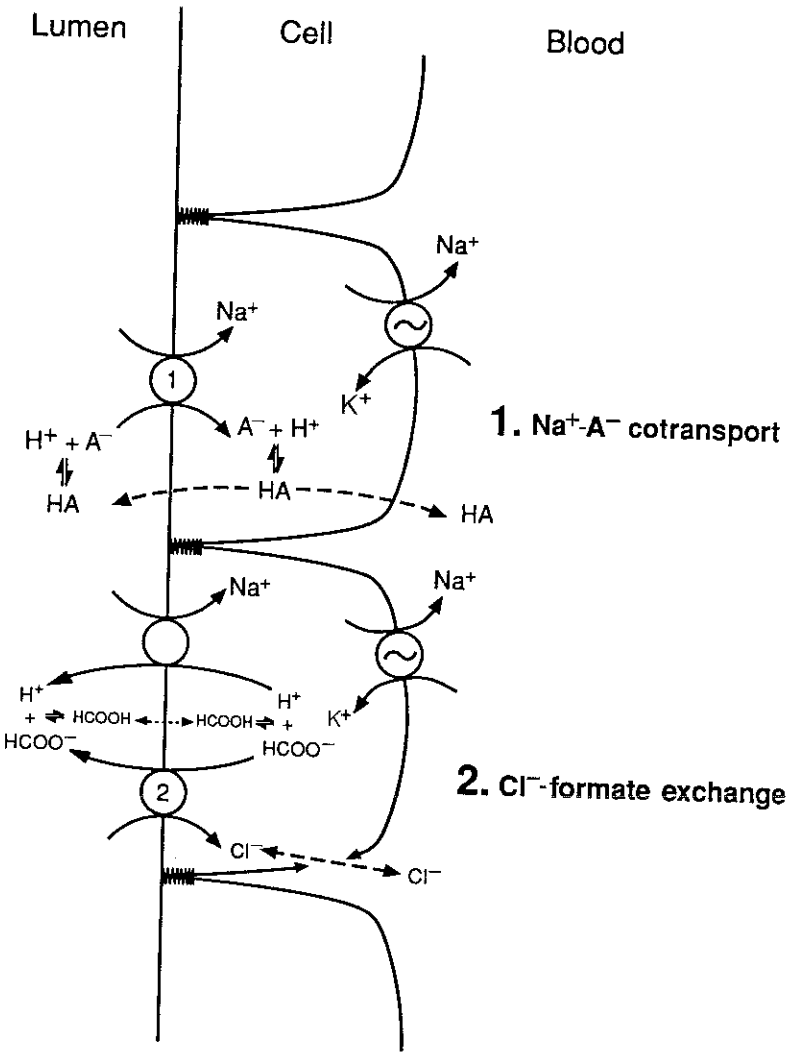

FIGURE 6. A model of anionic weak base fluxes in the renal proximal tubule mediated by either cotransport with Na^+ (1) or in exchange for Cl^- (2). An example of 1 is the Na^+-acetate cotransporter. The Cl^--formate exchanger is depicted by 2. These transporters are apparently involved in the luminal entry of Na^+ or Cl^- in NaCl-reabsorbing epithelia like the renal proximal tubule. Solid lines represent ion fluxes mediated by membrane transporters. Dotted lines represent passive diffusion of molecules across the membrane. Cl^- movements across the basolateral membrane are mediated by an ion-selective Cl^- channel.

of a Na^+ with an organic anionic weak base, such as lactate or acetate. Such cotransport would cause cellular alkalinization due to the entry of base. The anionic base would bind a proton upon entry and alkalinize the cell. These transport systems are likely to be part of a mechanism designed for the transepithelial movement of organic molecules and should be considered to **affect** pH_i rather than **regulate** it.

E. Chloride-Organic Anion Exchange

Another class of organic anion transporters has been described in renal proximal tubule cells. These transporters involve the exchange of organic anions for inorganic anions such as Cl^- or OH^-. For example, as part of the mechanism for NaCl reabsorption, an exchanger that mediates Cl^- influx (from the lumen) in exchange for formate efflux resides in the apical membrane of proximal tubule epithelia. This **Cl^--formate exchanger** (model 2 in Fig. 6) can be inhibited by disulfonic stilbene derivatives and is functionally similar to the Cl^--HCO_3^- exchanger. However, the Cl^--formate exchanger is distinct from the Cl^--HCO_3^- exchanger. The operation of this transporter during NaCl reabsorption should result in epithelial cell acidification. Other transporters involve the movement of organic anions including urate and oxalate. When these anions are transported into the cell, they will result in cellular alkalinization.

VII. Cellular Functions Affected by Intracellular pH

A wide variety of cellular processes and properties are affected by intracellular pH, and perhaps in some way all cell functions are influenced by the level of pH_i. It is impossible

to discuss all of the various effects, but many of the most important will be highlighted in the following sections (Fig. 7).

A. Cellular Metabolism

The fact that cellular metabolism can affect pH_i was discussed previously (see Section V.A). It has been appreciated for many years that the converse is also true; that is, changes of pH_i can affect cellular metabolism. Theoretically, because pH will affect the charge on ionizable groups in proteins, it would be anticipated that changes in pH_i could change the configuration of proteins and affect their activity. Such an effect of pH_i has been well documented for two key metabolic enzymes. Phosphofructokinase, a key glycolytic enzyme that converts fructose 6-phosphate (F6P) to fructose 1,6-diphosphate (FDP), has an exquisite pH sensitivity in the physiological range (6.5–7.5), its activity decreasing with a decrease of pH_i. The actual pH sensitivity is dependent on the cellular levels of F6P and 5′-AMP. Similarly, the conversion of phosphorylase (which catalyzes the metabolism of glycogen) from its inactive to active form is inhibited by a decrease in pH_i.

Two observations can be made from these findings. First, the pH dependence of enzyme activity is often affected by the concentrations of other factors, including substrates and other effectors. Thus, caution must be exercised in relating the *in vitro* pH profile of an enzyme to cellular conditions. Second, the general reaction of metabolic enzymes to a decrease in pH is a reduction in activity. This suggests that a decrease in pH_i could be used to prevent growth or to put a cell in a dormant state, as has been observed for many cells (see Section VII.G).

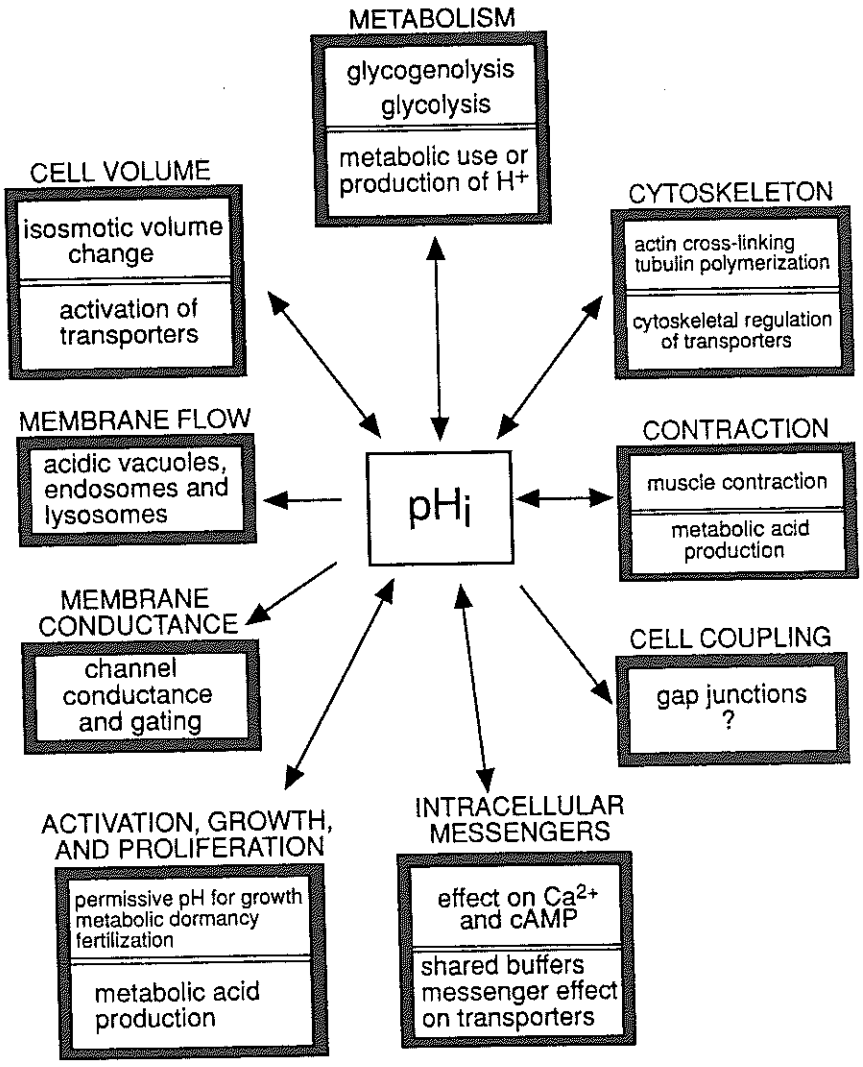

FIGURE 7. A summary of the cellular processes affected by intracellular pH. Each box represents a different cellular process. A two-headed arrow indicates that pH_i affects the process and that the process can also affect pH_i. Single-headed arrows suggest that pH affects that cellular function but that the cellular process probably does not have a major impact on pH_i. Within a box, the top half indicates the cellular processes affected by pH_i, while the bottom half indicates the mechanisms by which the process can affect pH_i.

B. Cytoskeleton

Changes in pH_i have been shown to affect the cytoskeleton, which can lead to changes in cell shape or motility. One example of such an effect on the cytoskeleton is the pH dependence of **actin filament** cross-linking to form gels. This cross-linking is mediated by actin-binding proteins whose ability to interact with actin is pH-dependent. In some cases, cell alkalinization increases the actin cross-linking to form a gel state in the cytoplasm or to form networks of microfilament bundles. In other cells, alkalinization reduces the cross-linking of actin filaments. The cell-specific responses of cytoskeletal cross-linking are probably due to different pH profiles of actin-binding proteins from different cells.

Changes in cellular pH can also affect the polymerization of cytoskeletal elements, such as **tubulin**. It has been shown that in some cells alkalinization can cause depolymerization of tubulin and disaggregation of microtubules within the cell. There are probably many other pH-dependent components to cytoskeletal assembly and function. It should be pointed out, however, that the conditions that lead to a change in pH_i are often accompanied by changes in intracellular calcium concentration and by phosphorylation of the cytoskeleton, and it is not always clear which is the predominant effector of cytoskeletal changes. Nevertheless, it is clear that changes in pH_i can play a major modulatory role in at least some alterations of the cytoskeleton.

C. Muscle Contraction

Intracellular acidification is known to reduce the ability of contractile cells to generate tension. This effect is particularly marked in cardiac muscle, but skeletal muscle also shows a reduced contractility at acidic values of pH_i. There are several possible ways by which cellular acidification could influence contractility: changes in surface channels could reduce cellular excitability, low pH could prevent calcium release from the sarcoplasmic reticulum through the calcium release channel, protons could compete with calcium for binding to the regulatory protein troponin, protons could inhibit the myofibrillar ATPase, or acidification could impair the ability of the cell to generate ATP. The effect of pH on muscle contraction could also be indirect. For example, during intense muscle activity inorganic phosphate (P_i) accumulates in the cell. A reduced pH will increase the diprotonated form of P_i ($H_2PO_4^-$), which has been shown to be particularly effective in inhibiting muscle force development during muscle fatigue.

D. Cell-Cell Coupling

The coupling of cells through **gap junctions** is apparently affected by intracellular pH. Several studies have suggested that a fall in pH_i uncouples gap junctions, thereby eliminating cell-cell coupling. The direct role of pH_i in the uncoupling process has been questioned, and it appears that changes in intracellular Ca^{2+} levels are responsible for uncoupling in some cells. The control of gap junction conductance may differ from cell to cell, but even in cells where Ca^{2+} is the primary regulator, changes in pH_i probably still have a modulatory effect on gap junction conductance.

E. Membrane Conductance

Ion-selective channels require the presence of charges within the channel proteins for proper ion conduction and channel gating. If these charges have p*K* values within the physiological range, these channels could well be affected by changes in pH. Indeed, the conductances of many channels are affected by changes in either pH_o or pH_i, including the tetrodotoxin-sensitive Na^+ channel, the delayed-rectifying and inward-rectifying K^+ channels, and Cl^- channels. Through the effect on conductance of membrane channels, changes in pH can affect the excitability of nerve and muscle cells and alter the membrane potential in all cells.

Recently, an interesting pH-sensitive K^+ channel has been described, the TASK channel (for TWIK-related acid-sensitive K^+ channel). The TASK channel exhibits rectification in asymmetric K^+ solutions that is consistent with the Goldman-Hodgkin-Katz equation, is noninactivating, is not voltage sensitive, and is highly sensitive to changes in **external** pH, with channel conductance falling with decreased pH_o. This type of leak channel is believed to be partly responsible for the resting membrane potential (especially in pancreas, placenta, and brain cells) and could explain the relationship between changes of pH_o and membrane potential in these cells.

F. Intracellular Messengers

Changes in pH_i can affect the levels of important intracellular signaling molecules, such as **Ca^{2+}** and **cAMP.** There are several possible ways by which pH can affect intracellular Ca^{2+}. An elevation of cytoplasmic H^+ can activate mitochondrial Ca^{2+}-H^+ exchange, resulting in a sequestering of H^+ within the mitochondria and an elevation of cytoplasmic $[Ca^{2+}]$. A decreased pH_i can reduce Ca^{2+} entry across the plasmalemma. The most direct interaction between cytoplasmic H^+ and Ca^{2+} ions, however, results from shared buffers. Many molecules that buffer H^+ will also bind, and thus buffer, Ca^{2+}. Depending on the relative affinities, an elevation of cytoplasmic $[H^+]$ can elevate intracellular $[Ca^{2+}]$ by displacing Ca^{2+} ions from intracellular buffer sites.

The interaction between changes of pH_i and $[Ca^{2+}]_i$ can also be indirect. An example of such an interaction is the pH-dependence of the binding of Ca^{2+} to calmodulin. Under certain conditions, a decrease in pH can be shown to reduce the binding of Ca^{2+} to calmodulin. Another example of the interaction between pH and Ca^{2+} is the pH-dependence of the interaction of the Ca^{2+}-calmodulin complex with other proteins, the direction of which depends on the protein being considered. Thus, the potential exists for changes of pH_i to alter the effect of Ca^{2+} on cellular function.

Changes of pH_i could affect another important intracellular signaling pathway as well, that involving cAMP. The proposed effects of pH_i on cAMP are based on the pH dependence of adenylyl cyclase (AC—the enzyme that synthesizes cAMP) and the cyclic nucleotide phosphodiesterase (PDE—the enzyme that hydrolyzes cAMP). In most cells, PDE apparently has a rather constant activity over the physiological range of pH (6.5–7.5). However, depending on the cell, an increase in pH can either markedly increase or de-

crease AC activity. Thus, alkalinization can result in either an increase or a decrease in cellular cAMP levels.

Given the pervasive effect of changes of pH_i on proteins, it is likely that pH_i effects on other signaling pathways, such as those mediated by cGMP or phosphoinositide metabolism, also exist. It should be noted, however, that the physiological significance of these pH_i effects on signaling pathways is often not clear, especially for cells that normally should see only small fluctuations of pH_i.

Some cells in the body function to sense changes in external pH, either in direct response to external acid (acid-sensing taste bud cells) or to elevated external CO_2 (glomus cells of the carotid body and central chemosensitive neurons). In these cells, extracellular acidification results in a **maintained** intracellular acidification, with pH recovery mechanisms being inhibited (most likely by decreased pH_o). It is believed that this decrease in pH_i inhibits K^+ channels, resulting in cell depolarization and increased generation of action potentials. Thus, in these chemosensitive cells, a decrease in pH_i apparently acts as an intracellular signal.

G. Cell Activation, Growth, and Proliferation

One of the most active areas of research on the role of intracellular pH in cell function has been the study of the role of changes of pH_i early in cell proliferation. These studies grew out of early observations that, shortly after fertilization of sea urchin eggs, egg pH increased markedly (roughly 0.4 pH unit), and this rise in pH was necessary for the initiation of growth by fertilization. These observations were followed by others on mammalian cells showing that a variety of growth-promoting agents, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, vasopressin, and serum albumin, similarly induced a cellular alkalinization (of about 0.1–0.2 pH unit) shortly after exposure. All of these alkalinizing effects are mediated by activation of Na^+-H^+ exchange. These growth-promoting agents activate the exchanger by activating cellular signaling pathways, which increase the affinity of the exchanger for internal H^+ ions and increase its activity, thereby alkalinizing the cell (see arrow 1 in Fig. 3).

It was initially hypothesized that the growth factor-induced increase in pH was part of a suite of early signals that are required for initiation of cell growth and proliferation. Cellular alkalinization was believed to contribute to the initiation of growth by activating key cellular enzymes that were then either direct effectors of growth (e.g., metabolic enzymes) or activators of other systems.

The direct signaling role of increases of pH_i in cell activation has been questioned. Changes in pH_i by themselves do not promote cell growth or division. Further, it has been shown that a number of cells have a higher pH_i in the presence of 5% CO_2 than in its absence. However, most of the initial experiments on the pH_i responses to growth factors had been done in the absence of CO_2. Upon repeating a number of these experiments under conditions more similar to physiological conditions (presence of 5% CO_2), the initial alkalinization upon exposure to stimulatory factors was not always seen, and in some cells the initial response was indeed an **acidification.** Thus, the current view of the role of pH_i in cell activation, growth, and proliferation is that pH_i plays a permissive role. That is, cells will only grow and proliferate if pH_i is above a certain critical value, regardless of how that value is obtained. If a cell had a pH_i above the critical value before exposure to a stimulatory agent, no change in pH_i would be required for cell growth. In fact, the cell could acidify and still grow as long as its pH_i stays above the critical value.

In reality, the role of pH_i as a signal to initiate cell growth may depend on the cell and the activating agent. It is likely that a rapid and marked rise in pH_i is one of the critical early steps necessary for the initiation of growth in fertilized sea urchin eggs. Even more dramatic is the nearly 1-pH-unit increase of pH_i in *Artemia* (brine shrimp) embryos upon arousal from anaerobic dormancy by exposure to oxygen. Undoubtedly, this large rise in pH_i is crucial for the transition from metabolic dormancy in these organisms. On the other hand, in many mammalian cells, the rather modest increase in pH_i on exposure to an activating agent is probably of limited physiological significance, especially given the variability in the degree and direction of pH changes observed in different experimental conditions.

Finally, even under conditions where a change of pH_i is not observed in response to a stimulatory agent, pH-regulating transport systems can still be shown to be activated. It has been hypothesized that although a change of pH_i may not be crucial for the stimulation of cell growth, the initiation of this growth may confront the cell with an acid or alkaline load. In this regard, activation of the pH-regulating transport systems by growth-promoting agents could be viewed as preparatory, enabling the cell to better maintain a constant pH_i during a period of high metabolic activity.

H. Cell Volume Regulation

Most pH-regulating transporters move ions such as Na^+ and Cl^- in exchange for a proton equivalent (H^+ or HCO_3^-). Because the transported proton equivalents are buffered (H^+ by HCO_3^- and protein buffers and HCO_3^- by formation of CO_2), they are osmotically "invisible." For example, virtually all of the H^+ transported by the Na^+-H^+ exchanger derive from internal buffers and upon efflux from the cell are buffered by external buffers. Thus, the Na^+-H^+ exchanger mediates the net import of one osmotically active particle (Na^+), and this import of osmolytes will be accompanied by the influx of water and cell swelling. Therefore, the Na^+-H^+ exchanger, in addition to contributing to pH_i regulation, can mediate the regulation of cell volume.

Other pH-regulating transporters can similarly mediate cell volume changes. For example, the Cl^--HCO_3^- exchanger transports Cl^- into the cell. The HCO_3^- that leaves combines with an H^+ and is removed as CO_2. Thus, like Na^+-H^+ exchange, Cl^--HCO_3^- exchange contributes to cell swelling. In fact, these two exchangers often act in concert to result in the net influx of NaCl (and therefore water) into the cell. The Na^+-HCO_3^- and Na^+-anionic weak base cotransporters would be ideally suited to mediate net solute transfer and therefore cell volume change. Finally, the (Na^++HCO_3^-)-Cl^- exchanger should not contribute to cell volume regulation because it mediates the entry of one osmotically active ion (Na^+) for the efflux of another (Cl^-).

Cell volume can be rapidly changed by exposure to anisosmotic media, with hypertonic media causing cell shrinkage, and hypotonic media causing cell swelling. Many cells respond to shrinkage with a **regulatory volume increase (RVI)** that involves the net uptake of solutes, and therefore water, so that cells swell back toward the initial cell volume (Fig. 8). In several different types of cells, RVI has been shown to involve an activation of Na^+-H^+ exchange. This exchanger, often in association with the Cl^--HCO_3^- exchanger, results in NaCl influx and a regulatory volume increase. The mechanism by which cell shrinkage activates the Na^+-H^+ exchanger is not fully understood, but interestingly, unlike most other activation pathways, cell shrinkage apparently does not result in phosphorylation of the exchanger. A possible involvement of the cell cytoskeleton in activating the Na^+-H^+ exchanger upon cell shrinkage is currently being investigated.

Recently, it has been shown that shrinkage can also activate (Na^++HCO_3^-)-Cl^- exchange in some cells. This observation is interesting since, as stated previously, this exchanger does not mediate any net solute movement and thus should not directly contribute to cell volume regulation. This activation of (Na^++HCO_3^-)-Cl^- exchange by cell shrinkage suggests that pH-regulating transporters may be activated by shrinkage to alkalinize the cell regardless of whether they contribute to volume regulation. It is not clear what benefit a shrunken cell derives from becoming alkaline, but it may involve pH-dependent cytoskeletal rearrangements (see Section VII.B).

In response to swelling, most cells exhibit a **regulatory volume decrease (RVD).** RVD involves the efflux of solutes accompanied by water and therefore cell shrinkage back toward the initial cell volume (Fig. 8). In at least one cell type, the nucleated red blood cell, RVD has been shown to be mediated by K^+-H^+ exchange (K^+ efflux and H^+ influx) in association with Cl^--HCO_3^- exchange (Cl^- efflux and HCO_3^- influx).

Cell volume can also be altered under isosmotic conditions by an imbalance of solute influx and efflux. For instance, during periods of active pH recovery from acidification, the Na^+ influx mediated by the Na^+-H^+ exchanger could result in cell swelling. Thus, changes in pH_i and the response to them could result in an alteration of cell volume.

It is clear that the regulation of intracellular volume and intracellular pH are highly linked in most cells. This linkage is due in part to the use of many of the same membrane transport systems for the regulation of cell pH and volume. In any given cell type, these transporters may respond predominantly either to changes in pH_i or to changes in cell volume.

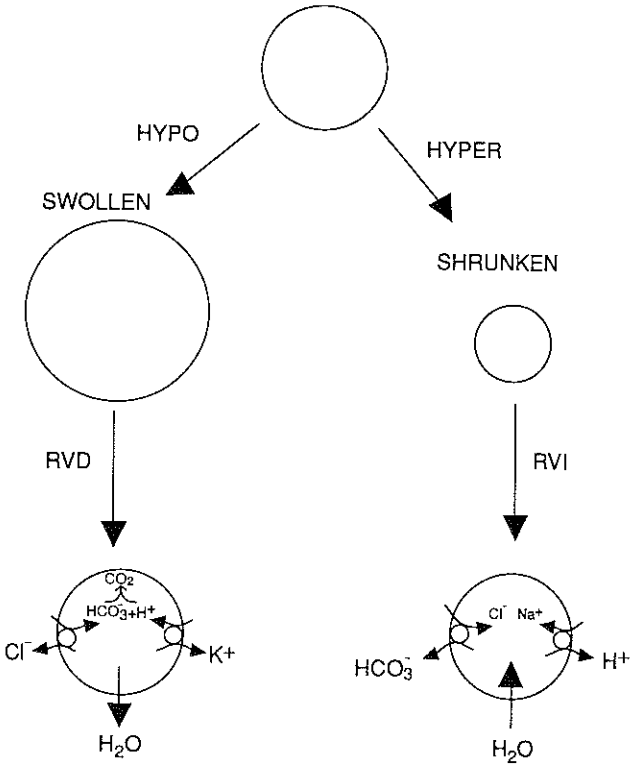

FIGURE 8. The role of pH-regulating transporters in cellular volume regulation. Upon cell swelling in hypotonic media, KCl has been shown to be removed from nucleated red blood cells by functionally coupled operation of the K^+-H^+ and the Cl^--HCO_3^- exchangers. The removal of KCl from the cell causes a loss of water from the cell and thus cell shrinkage in a process called regulatory volume decrease (RVD). Upon cell shrinkage in hypertonic media, NaCl enters the cell by parallel operation of Na^+-H^+ and Cl^--HCO_3^- exchangers. The NaCl entry results in water influx and cell swelling in a process called regulatory volume increase (RVI).

I. Intracellular Membrane Flow

The intracellular flow of membranes is affected by changes of pH within acidic vacuolar compartments. In cells, these vacuolar compartments are often involved in the movement of membranes, membrane-bound proteins, and soluble proteins around the cell. In addition, components of the vacuolar system are involved in the synthesis, processing, and degradation of various proteins. This system includes the endoplasmic reticulum, the Golgi apparatus, lysosomes, and endosomes. Movement of materials through this system can be divided into the endocytic and exocytic pathways. The endocytic pathway is involved in the uptake of external macromolecules, the degradation or delivery to the cell of these macromolecules, and the down-regulation of surface proteins; it includes coated pits, endosomes, and lysosomes. The exocytic pathway delivers newly-synthesized proteins to a variety of sites, including the surface membrane or extracellular space. Many of the compartments within this vacuolar system are acidic (see Section IV. B), and the maintenance of an acidic interior is critical for the functioning of these compartments. This criticality has been shown by the marked disturbance of endocytic and exocytic pathways by a number of agents, such as chloroquine and ammonia, which alkalinize these compartments. In addition, inhibition of vacuolar H^+-ATPase results in alkalinization of the acidic compartments and can lead to inhibition of endocytosis and exocytosis. Thus the maintenance of a proper pH in acidic intracellular compartments is crucial for continued and proper intracellular membrane flow.

VIII. Summary

Virtually all of the H^+ ions within a cell are buffered by reversible binding to weak acids and bases, resulting in a low free H^+ ion activity. Therefore, the activity of free H^+ ions within the cytoplasm is usually expressed as cytoplasmic pH (pH_i), defined as $pH = -\log(a_H)$, which is a more convenient scale for molecules at low activities.

Cytoplasmic pH is an important aspect of the intracellular milieu and can affect nearly all aspects of cell function. In most cells, pH_i is maintained at a value of about 7.0, well alkaline with respect to the equilibrium pH_i, calculated on the assumption that H^+ ions are at equilibrium across the membrane. The fact that pH_i is alkaline to its equilibrium value creates a passive acidifying influx of H^+. In fact, most cells face a continuous acid load due not only to this acidifying influx but to metabolic acid production and leakage from internal acidic compartments as well. Such challenges to a stable pH_i can be blunted by cellular buffers, but the only way to fully regulate pH_i is through the activity of membrane-bound transporters. These transporters fall into five categories: (1) cation-H^+ exchangers, such as the alkalinizing Na^+-H^+ exchanger and the acidifying K^+-H^+ exchanger; (2) HCO_3^--dependent transporters, such as the $(Na^+ + HCO_3^-)$-Cl^- and the Cl^--HCO_3^- exchangers and the Na^+-HCO_3^- cotransporter; (3) H^+-ATPases or proton pumps; (4) Na^+-organic anion cotransporters; and (5) Cl^--organic anion exchangers.

Changes in pH_i can affect many cellular functions. Cell metabolism can be affected by changes in pH, predominantly because of pH-sensitive metabolic enzymes, such as phosphofructokinase. Changes of pH_i have also been shown to affect the cross-linking and polymerization of cytoskeletal elements such as actin and tubulin. The loss of the ability of muscle cells to generate tension (muscle fatigue) has been correlated with a decrease of pH_i. Cell pH is also believed to have a modulatory role in gap junctions and many ion-selective channels. Further, changes of pH_i, mediated by activation of the Na^+-H^+ exchanger, may serve as an intracellular signal for the promotion of cell growth and proliferation. It is significant that changes in pH_i can affect other intracellular signals, such as cellular Ca^{2+} and cAMP levels, suggesting a complex interaction among cellular signaling systems.

Many pH-regulating transporters move an osmotically active ion, such as Na^+ or Cl^-, in exchange for a buffered (and thus osmotically "invisible") ion like H^+ or HCO_3^- and thus mediate the net movement of solute into or out of the cell. This net movement of solute will be accompanied by a net water flow and result in a change in cell volume. Thus, many pH-regulating transporters, in addition to contributing to pH_i regulation, can mediate the regulation of cell volume.

The pH of certain organellar compartments can differ from the value of pH_i and these differences in pH are important for organellar function. For example, mitochondria maintain an internal pH of 7.5, about 0.5 unit more alkaline than pH_i. This pH gradient across the mitochondrial membrane is essential for the major function of mitochondria, the production of ATP. Further, several intracellular organelles in the vacuolar system (e.g., endosomes, lysosomes, and storage granules) maintain an internal pH of 5–6, well below pH_i. These organelles contribute to the movement of membranes, membrane-bound proteins, and soluble proteins around the cell and their acidic pH is essential for this function.

Given the importance of pH to so many cellular functions, it is not surprising that cells have elaborated highly regulated mechanisms to control pH_i.

Bibliography

Al-Awqati, Q. (1986). Proton-translocating ATPases. *Annu. Rev. Cell Biol.* **2,** 179–199.

Ammann, D., Lanter, F., Steiner, R. A., Schulthess, P., Shijo, Y., and Simon, W. (1981). Neutral carrier based hydrogen ion selective microelectrode for extra- and intracellular studies. *Anal. Chem.* **53,** 2267–2269.

Aronson, P. S., and Boron, W. F. (Eds.). (1986). "Na^+-H^+ Exchange, Intracellular pH, and Cell Function," Vol. 26 in "Current Topics in Membranes and Transport," Academic Press, New York.

Aronson, P. S. (1989). The renal proximal tubule: a model for diversity of anion exchangers and stilbene-sensitive anion transporters. *Annu. Rev. Physiol.* **51,** 419-441.

Aronson, P. S., Nee, J., and Suhm, M. A. (1982). Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature* **299,** 161–163.

Attaphitaya, S., Park, K., and Melvin, J. E. (1999). Molecular cloning and functional expression of a rat Na^+/H^+ exchanger (NHE5) highly expressed in brain. *J. Biol. Chem.* **274,** 4383–4388.

Baird, N. R., Orlowski, H., Szabo, E. Z., Zaun, H. C., Schultheis, P. J., Menon, A. G., and Shull, G. E. (1999). Molecular cloning, genomic organization, and functional expression of Na^+/H^+ exchanger isoform 5 (NHE5) from human brain. *J. Biol. Chem.* **274,** 4377–4382.

Bell, S. M., Schreiner, C. M., Schultheis, P. J., Miller, M. L., Evans, R. L., Vorhees, C. V., Shull, G. E., and Scott, W. J. (1999). Targeted disruption of the murine Nhe1 locus induces ataxia, growth retardation, and seizures. *Am. J. Physiol.* **276,** C788–C795.

Bidani, A., and Brown, S. E. S. (1990). ATP-dependent pH_i recovery in lung macrophages: evidence for a plasma membrane H^+-ATPase. *Am. J. Physiol.* **259,** C586–C598.

Bock, G., and Marsh, J. (Eds.). (1988). "Proton Passage across Cell Membranes," Ciba Foundation Symposium 139, Wiley, New York.

Boron, W. F. (Ed.). (1986). Special topic: acid/base physiology. *Annu. Rev. Physiol.* **48,** 347–413.

Boron, W. F., and Boulpaep, E. L. (1983). Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. *J. Gen. Physiol.* **81,** 53–94.

Boron, W. F., and De Weer, P. (1976). Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *J. Gen. Physiol.* **67,** 91–112.

Busa, W. B., and Nuccitelli, R. (1984). Metabolic regulation via intracellular pH. *Am. J. Physiol.* **246,** R409–R438.

Cala, P. M. (1980). Volume regulation by *Amphiuma* red blood cells. The membrane potential and its implications regarding the nature of the ion-flux pathways. *J. Gen. Physiol.* **76,** 683–708.

Chamberlin, M. E., and Strange, K. (1989). Anisosmotic cell volume regulation: a comparative view. *Am. J. Physiol.* **257,** C159–C173.

Counillon, L., and Pouysségur, J. (1995). Structure-function studies and molecular regulation of the growth factor activatable sodium-hydrogen exchanger (NHE-1). *Cardiovascular Res.* **29,** 147–154.

DeCoursey, T. E. (1998). Four varieties of voltage-gated proton channels. *Frontiers in Biosci.* **3,** 477–482.

DeCoursey, T. E., and Cherney, V. V. (1994). Voltage-activated hydrogen ion currents. *J. Membr. Biol.* **141,** 1994.

Duprat, F., Lessage, F., Fink, M., Reyes, R., Heurteaux, C., and Lazdunski, M. (1997). TASK, a human background K^+ channel to

sense external pH variations near physiological pH. *EMBO J.* **16,** 5464–5471.

Durham, J. H., and Hardy, M. A. (Eds.). (1989). "Bicarbonate, Chloride, and Proton Transport Systems," Vol. 574 in "Annals of the New York Academy of Sciences." New York Academy of Sciences, New York.

Edmonds, B. T., Murray, J., and Condeelis, J. (1995). pH regulation of the F-actin binding properties of *Dictyostelium* elongation factor 1α. *J. Biol. Chem.* **270,** 15222–15230.

Gevers, W. (1977). Generation of protons by metabolic processes in heart cells. *J. Molec. Cell. Cardiol.* **9,** 867–874.

Grinstein, S. (Ed.). (1988). "Na^+/H^+ Exchange." CRC Press, Boca Raton, FL.

Grinstein, S. (1996). Non-invasive measurement of the luminal pH of compartments of the secretory pathway. *The Physiologist* **39,** 144.

Grinstein, S., and Rothstein, A. (1986). Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Membr. Biol.* **90,** 1–12.

Häussinger, D. (Ed.). (1988). "pH Homeostasis. Mechanisms and Control." Academic Press, New York.

Hille, B. (1992). "Ionic Channels of Excitable Membranes." Sinauer Associates, Sunderland, MA.

Hochachka, P. W., and Mommsen, T. P. (1983). Protons and anaerobiosis. *Science* **219,** 1391–1397.

Hoffmann, E. K., and Simonsen, L. O. (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69,** 315–382.

Karniski, L. P., and Aronson, P. S. (1985). Chloride/formate exchange with formic acid recycling: a mechanism of active chloride transport across epithelial membranes. *Proc. Natl. Acad. Sci. USA* **82,** 6362–6365.

Kopito, R. R., and Lodish, H. F. (1985). Primary structure and transmembrane orientation of the murine anion exchange protein. *Nature* **316,** 234–238.

Kotyk, A., and Slavik, J. (1989). "Intracellular pH and Its Measurement." CRC Press, Boca Raton, FL.

Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **95,** 6803–6808.

Lowe, A. G., and Lambert, A. (1983). Chloride-bicarbonate exchange and related transport processes. *Biochim. Biophys. Acta* **694,** 353–374.

Madshus, I. H. (1988). Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **250,** 1–8.

Masuda, A., Oyamada, M., Nagaoka, T., Tateishi, N., and Takamatsu, T. (1998). Regulation of cytosol-nucleus pH gradients by K^+/H^+ exchange mechanism in the nuclear envelope of neonatal rat astrocytes. *Brain Res.* **807,** 70–77.

Murer, H., Hopfer, U., and Kinne, R. (1976). Sodium/proton antiport in brush-border membranes isolated from rat small intestine and kidney. *Biochem. J.* **154,** 597–604.

Noël, J., and Pouysségur, J. (1995). Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *Am. J. Physiol.* **268,** C283–C296.

Nosek, T. M., Fender, K. Y., and Godt, R. E. (1987). It is diprotonated inorganic phosphate that depresses force in skinned skeletal muscle fibers. *Science* **236,** 191–193.

Nuccitelli, R., and Deamer, D. W. (Eds.). (1981). "Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Function." A. R. Liss, New York.

Palokangas, H., Metsikkö, K., and Väänänen. (1994). Active vacuolar H^+ ATPase is required for both endocytic and exocytic processes during viral infection of BHK-21 cells. *J. Biol. Chem.* **269,** 17577–17585.

Pouysségur, J., Sardet, C., Franchi, A., L'Allemain, G., and Paris, S. (1984). A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc. Natl. Acad. Sci. USA* **81,** 4833–4837.

Reusch, H. P., Lowe, J., and Ives, H. E. (1995). Osmotic activation of a Na^+-dependent Cl^-/HCO_3^- exchanger. *Am. J. Physiol.* **268,** C147–C153.

Rink, T. J., Tsien, R. Y., and Pozzan, T. (1982). Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol.* **95,** 189–196.

Ritucci, N. A., Chambers-Kersh, L., Dean, J. B., and Putnam, R. W. (1998). Intracellular pH regulation in neurons from chemosensitive and nonchemosensitive areas of the medulla. *Am. J. Physiol.* **275,** R1152–R1163.

Ritucci, N. A., Dean, J. B., and Putnam, R. W. (1997). Intracellular pH response to hypercapnia in neurons from chemosensitive areas of the medulla. *Am. J. Physiol.* **273,** R433–R441.

Romero, M. F., and Boron, W. F. (1999). Electrogenic Na^+/HCO_3^- cotransporters: cloning and physiology. *Annu. Rev. Physiol.* **61,** 699–723.

Roos, A., and Boron, W. F. (1980). The buffer value of weak acids and bases: origin of the concept, and first mathematical derivation and application to physico-chemical systems. The work of M. Koppel and K. Spiro (1914). *Respir. Physiol.* **40,** 1–32.

Roos, A., and Boron, W. F. (1981). Intracellular pH. *Physiol. Rev.* **61,** 296–434.

Sardet, C., Franchi, A., and Pouysségur, J. (1989). Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter. *Cell* **56,** 271–280.

Sardet, C., Counillon, L., Franchi, A., and Pouysségur, A. (1990). Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kD. *Science* **247,** 723–726.

Schultheis, P. J., Clarke, L. L., Meneton, P., Harline, M., Boivin, G. P., Stemmermann, G., Duffy, J. J., Doetschman, T., Miller, M. L., and Shull, G. E. (1998). Targeted disruption of the murine Na^+/H^+ exchanger isoform 2 gene causes reduced viability of gastric parietal cells and loss of net acid secretion. *J. Clin. Invest.* **101,** 1243–1253.

Schultheis, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gawenis, L. R., Riddle, T. M., Duffy, J. J., Doetschman, T., Wang, T., Giebisch, G., Aronson, P. S., Lorenz, J. N., and Shull, G. E. (1998). Renal and intestinal absorptive defects in mice lacking the NHE3 Na^+/H^+ exchanger.

Seksek, O., Biwersi, J., and Verkman, A. S. (1995). Direct measurement of *trans*-Golgi pH in living cells and regulation by second messengers. *J. Biol. Chem.* **270,** 4967–4970.

Seksek, O., and Bolard, J. (1996). Nuclear pH gradient in mammalian cells revealed by laser microspectrofluorimetry. *J. Cell Sci.* **109,** 257–262.

Soleimani, M., and Aronson, P. S. (1989). Ionic mechanism of Na^+-HCO_3^- cotransport in rabbit renal basolateral membrane vesicles. *J. Biol. Chem.* **264,** 18302–18308.

Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979). Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* **18,** 2210–2218.

Thomas, R. C. (1974). Intracellular pH of snail neurones measured with a new pH-sensitive glass micro-electrode. *J. Physiol. (London)* **238,** 159–180.

Thomas, R. C. (1978). "Ion-sensitive Intracellular Microelectrodes. How to Make and Use Them." Academic Press, New York.

Trivedi, B., and Danforth, W. H. (1966). Effect of pH on the kinetics of frog muscle phosphofructokinase. *J. Biol. Chem.* **241,** 4110–4111.

Wakabayashi, S., Ikeda, T., Iwamoto, T., Pouysségur, J., and Shigekawa, M. (1997). Calmodulin-binding autoinhibitory domain controls "pH-sensing" in the Na^+/H^+ exchanger NHE1 through sequence-specific interaction. *Biochemistry* **36,** 12854–12861.

Wakabayashi, S., Shigekawa, M., and Pouysségur, J. (1997). Molecular physiology of vertebrate Na^+/H^+ exchangers. *Physiol. Rev.* **77,** 51–74.

Yun, C. H. C., Tse, C. -M., Nath, S. K., Levine, S. A., Brant, S. R., and Donowitz, M. (1995). Mammalian Na^+/H^+ exchanger gene family: structure and function studies. *Am. J. Physiol.* **269,** G1–G11.

Appendix: Techniques for pH Measurement

The study of intracellular pH and its regulation was enabled by the development of the first reliable pH-sensitive microelectrode in 1974 by Roger Thomas, called the **Thomas recessed-tip microelectrode.** These electrodes are constructed from special glass that is conductive to H ions only. Small capillaries of this pH-sensitive glass are pulled into fine tip electrodes (tip diameter ≈ 1 μm) and these tips are sealed by heating. Such an electrode will respond with a Nernstian slope (about 59 mV/pH unit) to changes in pH. However, to reliably measure pH_i, one must assure that the pH-sensitive surfaces of this electrode are exposed to cytoplasm only. This is achieved by carefully lowering the sealed-tip pH electrode into a larger (about 2 μm) open-tip microelectrode (the shielding microelectrode) constructed from $AlSiO_4$ glass. The tip of the inner pH electrode is brought within a few micrometers of the open tip of the outer electrode (the distance BT, between tips in Fig. A-1). The pH electrode is heated, under internal pressure. The pH glass, which melts at a lower temperature than the $AlSiO_4$ glass, is pushed against the inner face of the shielding microelectrode and forms a high-resistance glass-glass seal (Fig. A-1). The inner electrode is removed above the seal and what remains is the tip of the pH electrode sealed near the tip of the shielding electrode (Fig. A-1). This recessed-tip electrode is filled with a buffered conducting solution and connected via a fine chlorided silver wire to an electrometer. The tip of the pH electrode below the seal (exposed length, EXPL in Fig. A-1) will respond to changes in the pH of the fluid trapped within the recess volume (striped area in Fig. A-1). When a cell is impaled with such an electrode, the fluid in the recess volume is replaced (by diffusion) with cytoplasm. A signal is generated across this electrode that is the sum of the actual membrane potential (V_m) and a Nernstian signal based on the pH difference between the electrode filling solution and the cytoplasm in the recess volume. To derive a signal that is directly proportional to the intracellular pH, a conventional open-tip KCl-filled microelectrode is placed within the same cell to measure V_m and the signals from the two electrodes are subtracted (Fig. A-2).

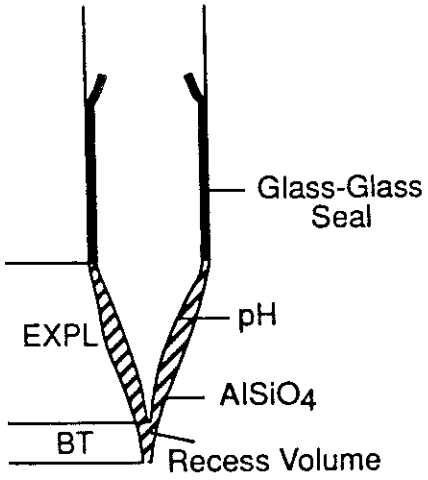

FIGURE A-1. A diagram of a pH-sensitive recessed-tip glass microelectrode showing the sealed tip of a microelectrode constructed from pH-sensitive glass fused within the tip of a larger shielding aluminosilicate electrode. BT—between-tips distance; EXPL—length of exposed pH-sensitive electrode below glass-glass seal.

The recessed-tip microelectrode gave some of the first continuous, reliable measurements of intracellular pH and initiated over two decades of intensive study of pH_i. However, this electrode has drawbacks. Because of its large size and the need to impale a cell with two electrodes, its use is restricted to fairly large cells. Further, the electrode has a high resistance (often greater than 100 GΩ) and must be used with a high input-impedance amplifier. Finally, because of the need for diffusional exchange between the recess volume and the cytoplasm, these electrodes have a slow response time (time constant no faster than 15 seconds).

A different type of pH microelectrode can be constructed using pH-sensitive resins (Fig. A-3). Like pH-sensitive glass, these resins are conductive to H ions only. A bit of this resin is placed in the tip of a conventional microelectrode and the electrodes are back-filled with a buffered conducting

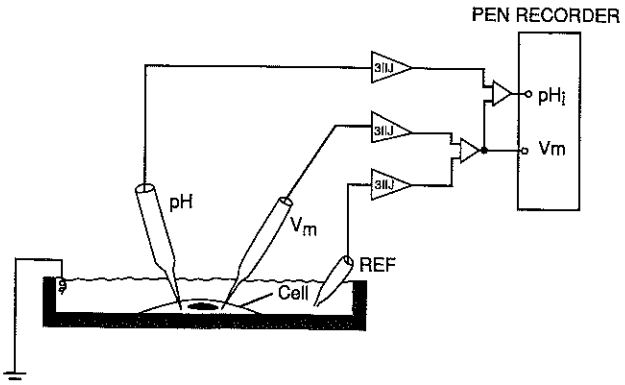

FIGURE A-2. Measurement of pH_i. Diagram of the apparatus used to measure intracellular pH with microelectrodes. The cell is impaled with a pH-sensitive and V_m-sensitive microelectrode. These electrodes are referenced to an open-tip flowing KCl reference electrode. Electrode voltages are amplified by Analog Devices 311J High Input Impedance Operational Amplifiers. The difference in electrode voltage between the V_m and reference electrodes is equal to membrane potential, and the difference in electrode voltage between the pH and V_m electrodes is a signal that is proportional to pH_i. These difference signals are plotted on a pen recorder.

medium (Fig. A-3). When a cell is impaled with these **pH-sensitive resin microelectrodes,** a signal is generated that is the sum of the V_m and a Nernstian signal due to the pH difference between the electrode and the cytoplasm. Thus a cell must be impaled with a V_m microelectrode when using the resin electrodes as well as the recessed electrodes. These pH-sensitive resin microelectrodes can penetrate much smaller cells because of their smaller tip diameters (usually less than 1 μm) and are faster than the recessed-tip electrodes. Further, pH-sensitive resin microelectrodes can readily be constructed in one barrel of a double-barreled electrode (the other barrel filled with KCl to measure V_m). Thus, the pH-sensitive resin microelectrodes have supplanted the recessed-tip microelectrode for most studies of pH_i.

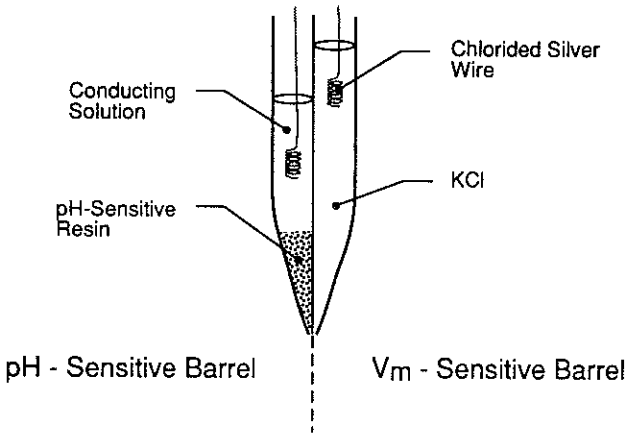

FIGURE A-3. A diagram of a double-barreled pH-sensitive resin microelectrode. The tip of one barrel of a double-barreled electrode is filled with a pH-sensitive resin and then back-filled with a conducting solution. This is the pH-sensitive barrel. The other barrel, the V_m-sensitive barrel, is filled with KCl. Each barrel is connected to an amplifier via a chlorided silver wire immersed in the conducting solution of that barrel.

Another reliable method for measuring intracellular pH is based on the fact that a number of dyes bind H ions reversibly and this binding affects the fluorescence of these dyes. The most common **pH-sensitive dye** in use today is a derivative of fluorescein, biscarboxyethyl carboxyfluorescein (BCECF) (Fig. A-4). An excitation spectrum for this dye can be obtained by shining light of different wavelengths (between 300 and 600 nm) on a solution containing this dye and measuring the fluorescence emitted at 535 nm. When such an excitation spectrum is gathered for the dye at different values of solution pH, the resulting spectra can be superimposed (Fig. A-5). Such curves reveal that when the dye is excited at 440 nm, its fluorescence is the same regardless of pH (called the isoexcitation point), whereas the dye is maximally sensitive to pH when excited at 500 nm, fluorescence increasing as pH increases.

The charged sites on the dye are often rendered neutral by attaching acetoxymethyl ester groups (BCECF/AM). In this form, the dye readily permeates the cell. Once inside, cell esterases cleave the AM groups from the dye, once again creating the charged form, BCECF. This form is impermeant and remains trapped within the cytoplasm. The fluorescence of this dye within cells can be measured using a spectrofluorimeter.

The dye within cells can slowly leak out or photobleach during the course of an experiment. Either of these processes would result in a decreased fluorescence signal and appear as a decrease in pH_i. To prevent this artifact, the dye fluorescence is often collected at excitation wavelengths of both 440 and 500 nm and a fluorescence ratio (R_{fl}), Fl_{500}/Fl_{440}, calculated. In this ratio, Fl_{440} is a measure of the amount of dye present and thus serves as a normalizing factor. R_{fl} is proportional to intracellular pH and is not susceptible to leakage or photobleaching artifacts. The fluorescence ratio can be calibrated by exposing cells to a high extracellular $[K^+]$ (similar to intracellular $[K^+]$) and nigericin, a K^+-H^+-exchanging ionophore. Under these conditions it is assumed that the nigericin will equilibrate intracellular and extracellular pH. Upon exposing dye-loaded cells to a variety of extracellular pH values and measuring R_{fl}, a calibration curve can be constructed (Fig. A-6). The use of pH-sensitive fluorescent dyes to study pH_i has several

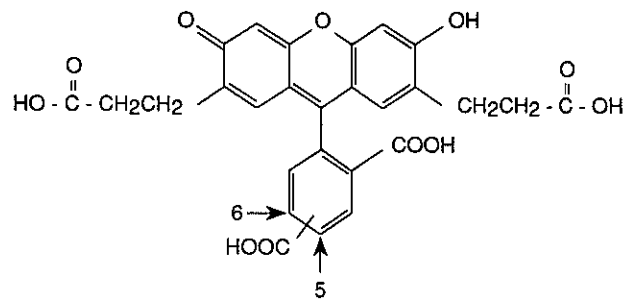

FIGURE A-4. A diagram of the pH-sensitive fluorescent probe BCECF.

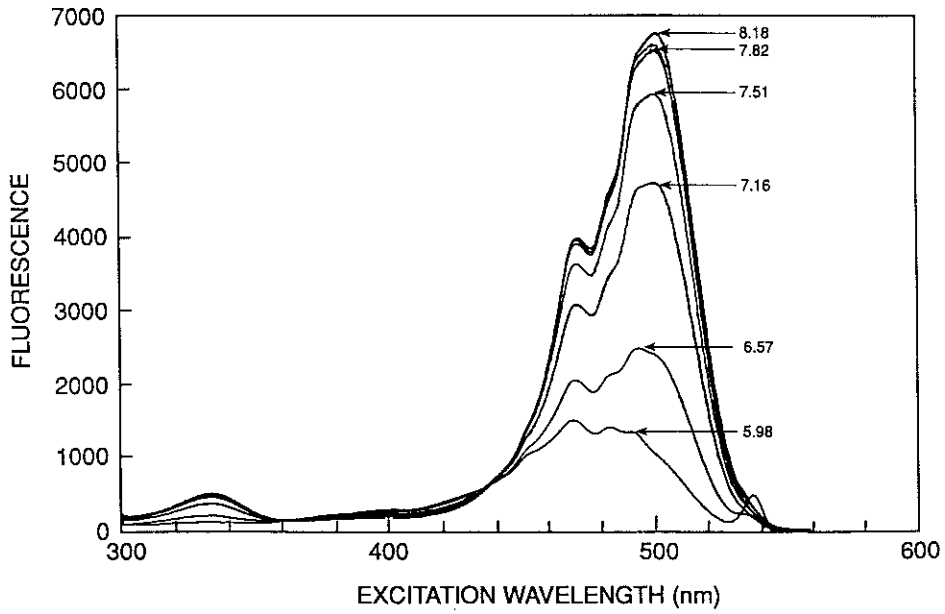

FIGURE A-5. Excitation spectra of BCECF: the fluorescent emission (at 535 nm) of BCECF when excited at wavelengths from 300–600 nm. Each curve represents the excitation spectrum of BCECF at a different pH (indicated to the right of each spectrum). The ordinate represents fluorescence in arbitrary units.

advantages over microelectrodes. This technique can be used on cells of any size, including preparations of intracellular organelles. Further, it is a relatively simple and reliable experimental technique. Finally, this technique can be used to visualize pH within single cells or parts of cells, using optical imaging techniques.

To study the ability of cells to actively extrude acid or base from the cell, techniques must be available to alter intracellular pH experimentally. In some large cells, this has been achieved directly by injecting acid into cells, by passing current through microelectrodes, or by internal dialysis through tubing threaded through the cytoplasm of the cell. More recently, changes in pH_i have been accomplished by internal perfusion of cells using whole-cell patch-clamp electrodes. However, the most commonly used experimental method to modify pH_i is by external exposure of cells to weak acids or bases. One of the most popular of such techniques is the **NH_4Cl prepulse technique.** Cells are exposed to an external solution containing NH_4Cl. External NH_3, being uncharged, enters the cell (arrow 1 in Fig. A-7A) far more rapidly than external NH_4^+. In the cell, the NH_3 combines with an H ion to form NH_4^+ (arrow 2 in Fig. A-7A), thereby alkalinizing the cell. This alkalinization will continue until the internal and external concentrations of NH_3 are the same, at which time no more NH_3 will enter and the pH_i will reach a new steady-state value alkaline relative to the original pH_i. If NH_4^+ is unable to enter the cell, no further change in pH_i will occur until external NH_4Cl is removed, at which time cytoplasmic NH_4^+ will dissociate to NH_3 and H^+, and all the NH_3 will diffuse from the cell, returning pH_i back to its original value (top pH trace in Fig. A-7A). However, if NH_4^+ has some membrane permeability, it will enter the cell (arrow 3 in Fig. A-7A), largely driven by the negative internal membrane potential. The NH_4^+ that enters will dissociate into NH_3 and H^+. The newly formed NH_3 will diffuse from the cell, leaving H^+ in the cell. Thus, a shuttle is established whereby NH_4^+ enters the cell and NH_3 leaves the cell (arrows 3, 2, 1 and 4 in Fig. A-7A). For each cycle of the shuttle, an H ion is added to the cell and so the cell slowly acidifies in the maintained presence of external NH_4Cl (termed *plateau acidification*). Upon removal of external NH_4Cl, all the original NH_4^+ formed upon exposure will dissociate and regenerate NH_3 (which diffuses from the cell) and H^+. However, the cell will contain extra H^+ due to the operation of the shuttle and thus pH_i will undershoot its initial value, achieving a more acid pH_i than it

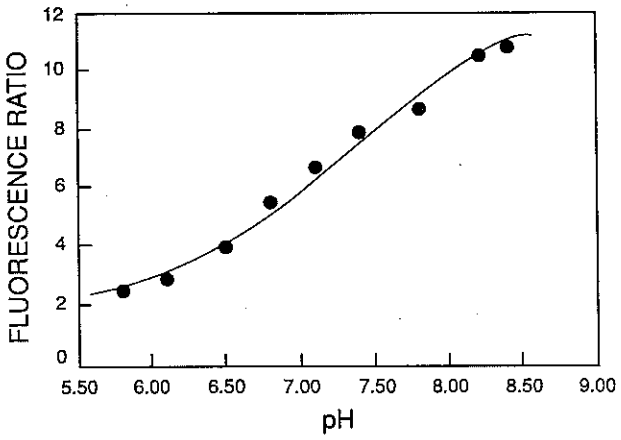

FIGURE A-6. A calibration curve for BCECF derived from data shown in Fig. A-5. The ratio of the emitted fluorescence at excitation wavelengths of 500 and 440 nm ($R_{fl} = Fl_{500}/Fl_{440}$) is plotted against the pH of the solution and a sigmoid titration curve is obtained.

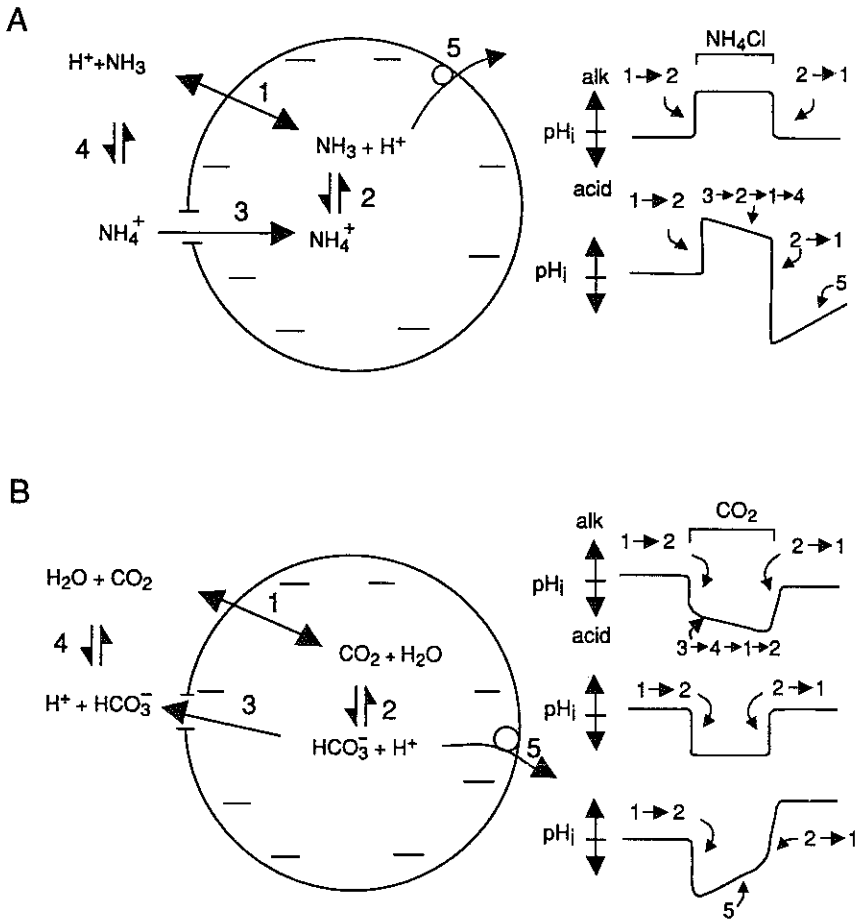

FIGURE A-7. (A) A diagram of the effects on intracellular pH of a transient exposure of a cell to external NH_4Cl. (B) A diagram of the effects on intracellular pH of a transient exposure of a cell to external CO_2 and HCO_3^-. The meaning of the various numbers and the pH traces to the right are given in the Appendix text.

had before the NH_4Cl exposure. The degree of this undershoot is dependent on the amount of NH_4Cl initially added, the membrane permeability to NH_4^+, and the duration of exposure to NH_4Cl. The net effect of an NH_4Cl prepulse is to acidify the cell, and it is entirely analogous to an injection of acid. If a cell possesses membrane transport systems for active H^+ extrusion (arrow 5 in Fig. A-7A), pH_i will return toward its initial pH value, a process termed **pH recovery** (lower pH trace in Fig. A-7A).

The other common way to alter pH_i is by exposure of cells to weak acids. The most common weak acid is carbonic acid, in the form of CO_2. This process is analogous to the NH_4Cl prepulse. Upon exposure to a solution containing CO_2 and HCO_3^-, CO_2 rapidly enters the cell (arrow 1 in Fig. A-7B), hydrates and dissociates to form internal HCO_3^- and H^+ (arrow 2 in Fig. A-7B). The addition of H^+ internally acidifies the cell. The cell will continue to acidify until internal and external CO_2 are equal. At this point, if HCO_3^- is impermeant, no further change in pH will occur, and pH will return to its initial value upon removal of extracellular CO_2 (middle pH trace in Fig. A-7B). However, if HCO_3^- can move across the membrane, a shuttle will be established. HCO_3^- will leave the cell (arrow 3 in Fig. A-7B), mostly driven by the negative membrane potential, internal CO_2 will hydrate and dissociate, and more CO_2 will enter the cell. Thus, HCO_3^- will leave the cell and CO_2 will enter the cell, adding an internal H^+ for every cycle of the shuttle (arrows 3, 4, 1 and 2 in Fig. A-7B). The cell will slowly acidify (top pH trace in Fig. A-7B). If, however, the cell possesses transmembrane H^+ extrusion mechanisms (arrow 5 in Fig. A-7B), pH will recover back toward the initial pH_i value because of active extrusion of internal H^+ even in the maintained presence of CO_2. If external CO_2 is removed after recovery, the H^+ initially formed upon CO_2 exposure will recombine with HCO_3^- and leave the cell as H_2O and CO_2, thereby alkalinizing the cell. However, the cell pH will overshoot, reaching an alkaline value of pH (bottom pH trace in Fig. A-7B), because of the removal of internal H^+ during pH recovery. Thus, exposure to or removal of weak acids can induce cellular acidification or alkalinization, respectively.