Because of the large number of biological processes sensitive to changes in pH (see reviews [88, 117, 456]), the subject of acid-base homeostasis has attracted considerable attention over the past several decades. Until relatively recently, acid-base homeostasis, for both clinicians and basic scientists, has been synonymous with pH regulation in the two most easily accessed compartments, blood and cerebrospinal fluid (CSF). The pH in these extracellular compartments (pHe) is certainly important for organisms. For example, alterations in pHe may affect various extracellular biochemical reactions (e.g., hemostasis, complement fixation) and influence binding of various substances (e.g., hormones, metals, therapeutic agents) to plasma proteins or cell surface receptors. Moreover, certain ion channels (524, 555) as well as transporters that move solutes across cell membranes are sensitive to pHe changes. Nevertheless, the number of processes sensitive to changes of extracellular pH pales in comparison with the myriad processes sensitive to alterations in intracellular pH (pHi). Thus, pHi homeostasis should be a matter of central importance not only for individual cells, but also for the organism composed of these cells.

Although cellular metabolism can modulate pHe, the regulation of pHi is the province of membrane proteins that transfer acid-base equivalents across the plasma membrane. In addition, transporters that carry acid-base equivalents across organellar membranes can transiently modify pHi, or can participate in the buffering of cytoplasmic H⁺.

Since the last edition of this book, the field of pHi regulation has advanced on two broad fronts: (1) elucidating the complex mechanisms responsible for pHi regulation and (2) understanding the molecular mechanisms of pHi regulation.

ELUCIDATING THE COMPLEXITY OF pH\textsubscript{i} REGULATION

One of the themes that continue to emerge is that patterns of pHi regulation in particular cell types tend to be unique and complex. As a result, without previous knowledge of a cell’s physiology, it may be impossible to predict its response to a particular maneuver. For example, switching the extracellular buffer from a non-CO\textsubscript{2}/HCO\textsubscript{3}⁻ buffer to CO\textsubscript{2}/HCO\textsubscript{3}⁻ usually causes an abrupt fall in pHi, due to influx of the highly permeant CO\textsubscript{2}. On the other hand, some cell membranes show no evidence whatsoever of being permeable to gases such as CO\textsubscript{2} (573) or NH\textsubscript{3} (289, 511, 573). In fact, some of the CO\textsubscript{2} permeability of membranes may require “gas” channels (126, 188, 392, 427). For cells with CO\textsubscript{2}-permeable membranes, the response to the initial CO\textsubscript{2}-induced acidification may—depending on the cell type and initial pHi—be a pHi recovery that is totally absent (59), partial (591), complete (545), or even excessive (6, 49, 53, 75, 104, 151, 391, 431).

pHi regulation is not only diverse, it is also complex. Thus, a particular cell type may possess numerous plasma-membrane transporters that regulate pHi, each with its own unique properties. Since the last edition of this book, many pHi-regulating mechanisms have been functionally studied in greater detail. These mechanisms include Na\textsuperscript{+}/HCO\textsubscript{3}⁻ cotransporters.

pHi regulation is also complex in that the Na\textsuperscript{+}–H\textsuperscript{+} exchanger and other acid-base transporters may be under the concerted control of growth factors (104, 199–202, 381) or other environmental influences, such as acidosis (428) or...
hypertonicity (397). A G-protein–coupled H⁺ receptor has been described (345). In the proximal tubule, the basolateral concentration of [CO₂] and [HCO₃⁻] per se—indeed, independent of basolateral pH—are powerful regulators of acid-base transport (618).

Given the complexity and diversity of patterns of pH, regulation, one must examine pH, physiology anew for each previously unexplored cell type.

UNDERSTANDING THE MOLECULAR MECHANISMS OF pH, REGULATION

Since the last edition of this book, the field has seen major advances in the molecular biology of acid-base transporters. One of the advances has been in organizing the vast array of proteins responsible for transport. The Human Genome Organization (HUGO) created the SLC superfamily of solute carriers, which currently contains 43 families. Three of these contain members that pH physiologists have traditionally regarded as pH,–regulated proteins: the SLC4 family of HCO₃⁻ transporters (618), the SLC9 family of Na⁺⁻H⁺ exchangers (405), and the SLC26 family of multi-functional anion exchangers (384). Table 1 summarizes these and 21 other SLC families that contain members that transport acid–base equivalents. Over the past several years, investigators have cloned cDNAs encoding new members of many of these families and have identified a myriad of splice variants of the SLC proteins listed in Table 1.

In addition to the SLC transport proteins, several other families of protein engage in acid–base transport, including vesicular H⁺ pumps, P-type ATPases (e.g., the gastric H⁺⁻K⁺ pump and the SERCA Ca²⁺⁻H⁺ pump), Cl⁻ channels that also mediate the flux of HCO₃⁻, H⁺ channels, and aquaporins that mediate the flux of CO₂ or NH₃.

Given the recent advances in our understanding of acid-base transport at the molecular level, the future holds great promise for (1) determining the molecular identity of transporters responsible for pH, regulation in a particular cell type, (2) studying transport function under well-defined conditions (e.g., transporters heterologously expressed in Xenopus oocytes or other cells), and (3) elucidating the structural basis of transporter function.

SCOPE OF THIS CHAPTER

In this chapter, we will first consider the methodologies available for making pH, measurements. We will then examine the factors that contribute to changes in pH, the thermodynamic forces acting on hydrogen ions (H⁺) and other charged acids/bases; the permeation of the cell membrane by electrically neutral acids and bases; the buffering power of the intracellular fluid; and transporters that regulate pH, by moving H⁺, bicarbonate ions (HCO₃⁻) and/or other weak acid/bases across the plasma membrane. Because space does not permit us to summarize the vast array of acid base transporters presented in Table 1, we will focus on transporters that play a major role in pH, regulation. Emerging from this discussion will be a model of pH, regulation. Finally, in light of this model, we will consider several factors that fundamentally alter pH, regulation: alterations in pH, temperature changes; metabolic inhibitors and hypoxia; cell shrinkage; hormones, growth factors, and oncogenes.

METHODS FOR MEASURING pH,

Of the techniques available for measuring pH, we will consider the four that are of greatest utility. These techniques differ from one another in terms of their theoretical foundations, the precise cellular properties that they measure, as well as in their accuracy and sensitivity.

pH-SENSITIVE MICROELECTRODES

In the mid 1990s, it was beginning to look like the use of pH microelectrodes might become a dying art, except for their restricted use for measuring pH, in large invertebrate cells.

TABLE 1  SLC Families with Members That Can Engage in Acid-Base Transport

<table>
<thead>
<tr>
<th>SLC Family Number</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High-affinity glutamate transporters</td>
</tr>
<tr>
<td>4</td>
<td>HCO₃⁻ transporters</td>
</tr>
<tr>
<td>5</td>
<td>Na/glucose cotransporters (which include Na/monocarboxylate cotransporters)</td>
</tr>
<tr>
<td>6</td>
<td>Na- and Cl-dependent neurotransmitter transporters</td>
</tr>
<tr>
<td>7</td>
<td>Cationic amino-acid transporters</td>
</tr>
<tr>
<td>9</td>
<td>Na-H exchangers</td>
</tr>
<tr>
<td>10</td>
<td>Na/bile-salt transporters</td>
</tr>
<tr>
<td>11</td>
<td>H⁺-coupled metal-ion transporters</td>
</tr>
<tr>
<td>13</td>
<td>Na-sulfate-carboxylate cotransporters</td>
</tr>
<tr>
<td>15</td>
<td>H⁺/oligopeptide cotransporters</td>
</tr>
<tr>
<td>16</td>
<td>H⁺/monocarboxylate cotransporters</td>
</tr>
<tr>
<td>17</td>
<td>Type I Na/phosphate cotransporter and vesicular glutamate transporters</td>
</tr>
<tr>
<td>18</td>
<td>Vesicular amine transporters</td>
</tr>
<tr>
<td>19</td>
<td>Folate/thiamine transporters</td>
</tr>
<tr>
<td>20</td>
<td>Type III Na/phosphate cotransporters</td>
</tr>
<tr>
<td>21</td>
<td>Organic-anion transporters</td>
</tr>
<tr>
<td>22</td>
<td>Organic cation/anion transporters</td>
</tr>
<tr>
<td>23</td>
<td>Na-dependent ascorbate transporters</td>
</tr>
<tr>
<td>24</td>
<td>Mitochondrial carriers</td>
</tr>
<tr>
<td>25</td>
<td>Multifunctional anion transporters</td>
</tr>
<tr>
<td>26</td>
<td>Fatty-acid transporters</td>
</tr>
<tr>
<td>27</td>
<td>Vesicular inhibitory-amino-acid (GABA) transporters</td>
</tr>
<tr>
<td>34</td>
<td>Type II Na/phosphate cotransporters</td>
</tr>
<tr>
<td>36</td>
<td>H⁺-coupled amino-acid transporters</td>
</tr>
<tr>
<td>42</td>
<td>Rh family (NH₃ transport)</td>
</tr>
</tbody>
</table>
However, the emergence of the *Xenopus* oocyte expression system and the cloning of numerous acid-base transporters over the past decade have breathed new life into an old technology.

When a pH electrode and an indifferent reference electrode are placed in a solution, the voltage difference between the electrodes (E	extsubscript{d}) is linearly related to the solution pH (pH	extsubscript{d}):

\[
\text{pH}_x = \text{pH}_i + (E_x - E_i) \frac{F}{RT\ln 10} \quad (\text{Eq. 1})
\]

Here, pH	extsubscript{i} is the pH of a standard solution, and E	extsubscript{i} is the voltage difference in this standard. F/(RT ln 10) is the theoretical slope (~58 mV per pH-unit change at 22°C) of the line relating pH to voltage. The actual slope is determined empirically.

Although the pH sensor can be any of several materials (e.g., platinum—hydrogen, antimony, tungsten, or a liquid membrane), the most reliable remains pH-sensitive glass. Glass has the advantages of long lifetime, long-term stability, and insensitivity to cations other than H\textsuperscript{+} (at physiological pH), redox reactions, and various gases. Several styles of glass pH-sensitive microelectrodes are available, including Hinke’s exposed-tip design (254), RC Thomas’ recessed-tip design (543), and RC Thomas’ eccentric design (488, 548). Glass electrodes are particularly well suited to measure pH, of relatively large cells (e.g., squid axons) or to measure pH, but are usually not feasible for small cells, glass electrodes generally have been abandoned in favor of microelectrodes with liquid-membrane sensors, which are easier to make and have a much shorter lifetime, are less stable and are sometimes sensitive to other parameters.

Ideally, the pH and reference microelectrodes must impale a single cell. An acceptable alternative may be to place them in two identical cells or in cells that are electrically coupled. Another solution is to use a double-barreled electrode (143, 359). Unfortunately, for each barrel, there is a trade-off between tip size and electrode performance. Thus, the larger the two orifices of a double-barreled electrode, the better the electrode performance, but the greater the cell damage caused by impalement. The performance of liquid-membrane electrodes can be improved by using a concentric design in which a saline-filled pipette is threaded into the column of liquid-membrane sensor, thereby reducing the overall longitudinal resistance between the sensor at the electrode’s tip and the electrical contact in the electrode’s barrel (174, 406, 561, 562). Fedirko et al. (174) have described a simplified approach for implementing this concentric design for H\textsuperscript{+} and Ca\textsuperscript{2+}-selective microelectrodes, permitting rapid measurements of extracellular pH and Ca\textsuperscript{2+} transients in rat hippocampal brain slices.

pH-sensitive electrodes, particularly those with glass sensors, remain the method of choice for monitoring pH, in relatively large cells. Not only are these electrodes highly sensitive (<0.01 pH units), they are probably more precise and accurate than pH-sensitive dyes. Moreover, the microelectrode reports pH and cell voltage simultaneously, and in real time. The “pH” that they report is almost certainly that of the bulk cytoplasm (i.e., the fluid in direct contact with the plasma membrane), uncontaminated by the pH of organelles. Disadvantages of using microelectrodes for measuring pH include the time and skill required for making and using them. In addition, one can only use conventional microelectrodes on cells large enough to sustain the impalement. Although small mammalian cells generally cannot tolerate such impalement, pH-sensitive microelectrodes placed near the extracellular side of the plasma membrane of a mammalian cell can be used to measure H\textsuperscript{+} fluxes due to acid-base transporter activity. Using an elegant “self-referencing” pH microelectrode technique with Chinese hamster ovary (CHO) fibroblasts, Fuster et al. (194) measured the continual extracellular H\textsuperscript{+} gradient when a cell-attached patch pipette was repetitively positioned close to and away from an extracellular pH electrode. H\textsuperscript{+} fluxes due to altered Na-H exchanger activity were quantitated from changes in the extracellular H\textsuperscript{+} gradient elicited by altering the cytoplasm via pipette perfusion.

## DISTRIBUTION OF WEAK ACIDS AND BASES

Cell membranes are generally far more permeable to neutral molecules than to charged molecules of similar shape and size. Thus, if a cell is exposed to a monoprotic weak acid HA (HA \rightleftharpoons H\textsuperscript{+} + A\textsuperscript{-}), the neutral molecule rapidly enters the cell (Fig. 1). Assuming for the moment that A\textsuperscript{-} cannot penetrate the membrane, the entry of HA continues until HA is in equilibrium across the cell membrane; that is, when the concentration of HA inside the cell ([HA]\textsubscript{i}) is the same as that outside ([HA]\textsubscript{o}). Because entering HA dissociates to H\textsuperscript{+} and A\textsuperscript{-}, the equilibrium of HA across the membrane is necessarily accompanied by a fall in pH. This principle underlies the pH changes caused by neutral weak acids and bases, which we will discuss below in Effects of Weak Acids and Bases on pH. Provided that the dissociation constant (K = [H\textsuperscript{+}]\times[A\textsuperscript{-}]/[HA]) is the same both inside and outside the cell, then, at equilibrium,

\[
[H^+]_i \times [A^-]_i = K \times [HA]_i,
\]

\[
= [H^+]_o \times [A^-]_o. \quad (\text{Eq. 2})
\]

and

\[
\frac{[H^+]_i}{[H^+]_o} = \frac{[A^-]_i}{[A^-]_o}. \quad (\text{Eq. 3})
\]

Because the transmembrane distribution ratios of A\textsuperscript{-} and H\textsuperscript{+} are inversely related, we can use Eq. 3 to compute pH,
FIGURE 1 The distribution of a monoprotic weak acid across a cell membrane. When placed in the external solution, HA passively enters the cell, where it dissociates to form H⁺ and A⁻. Once [HA] = [HA]₀, the equilibrium HA → H⁺ + A⁻ holds in both the intracellular and extracellular fluids.

The weak acid most commonly used to calculate pH is 5,5-dimethyl-2,4-oxazolidinedione (DMO), used as ¹⁴C-DMO; benzoic acid has also been used (160, 321). Regardless of the weak acid used, the amount of radioactivity in the intra- or extracellular fluid is proportional to the total concentration of the probe, [A⁻] + [HA]. Because it is impossible to measure [A⁻] directly, we must modify the foregoing equation to include the concentrations of the total probe:

\[
\frac{[A^-] + [HA]}{[A^-] + [HA]_0} = \frac{1 + K/[H^+]}{1 + K/[H^+]},
\]

(Eq. 4)

Equation 4 can then be solved for pH:

\[
\text{pH} = \text{pK} + \log \left( \frac{[A^-] + [HA]}{[A^-] + [HA]_0} \right) \left( 10^{\text{pH} - \text{pK}} + 1 \right)
\]

(Eq. 5)

One can use a similar approach to compute pH from the distribution of a permeant weak base, such as methylamine (67). Reviews by Waddell and Butler (570) and Roos and Boron (456) contain more detailed descriptions of the weak-acid/base method, including potential difficulties.

The major advantages of the weak-acid/base method include its technical simplicity and applicability to even very small cells. The parameter actually measured is not the pH of the cytoplasm, but rather a volume-weighted mean pH of all intracellular compartments in which the weak acid or base is distributed. The practical sensitivity of this approach is 0.03 to 0.05 pH units—considerably less than the microelectrode technique. The major disadvantage of the weak-acid/base approach is that continuous pH measurements are not possible.

**pH-SENSITIVE DYSES**

**Absorbance**

Molecules with an absorbance, fluorescence excitation and/or fluorescence emission spectrum sensitive to pH may be convenient probes for measuring pH. If one exposes a solution of a pH-sensitive dye to light, then dye molecules may absorb some of the light as electrons make the transition to a higher-energy state. From the intensities of incident light (Iᵢ) and the light transmitted through the solution (I), we can compute the absorbance (A):

\[
A = \log \frac{I_i}{I} \quad \text{(Eq. 6)}
\]

According to the Beer-Lambert law, A at a particular wavelength (λᵢ) is proportional to both the length of the light’s path through the solution (l) and the concentration of the dye (C):

\[
A = \epsilon_{λᵢ}\text{pH} \quad \text{(Eq. 7)}
\]

where the proportionality constant \(\epsilon_{λᵢ}\text{pH}\) is the dye’s extinction coefficient at a particular wavelength and pH. The wavelength dependence of \(\epsilon_{λᵢ}\text{pH}\) defines how this shape is affected by changes in pH. However, \(\epsilon_{λᵢ}\text{pH}\) depends not only upon pH, but also upon l and C. If we were to attempt to compute pH from absorbance data at a single wavelength, then we would need to know both l and C, two parameters that are in practice very difficult to ascertain in a live cell. Alternately, we could obtain the absorbance data at two wavelengths (λ₁ and λ₂), and compute the absorbance ratio \(A_{λ₁}/A_{λ₂}\):

\[
\frac{A_{λ₁}}{A_{λ₂}} = \frac{\epsilon_{λ₁}\text{pH}}{C} \quad \frac{\epsilon_{λ₂}\text{pH}}{C} = \frac{\epsilon_{λ₁}\text{pH}}{C} \quad \frac{\epsilon_{λ₂}\text{pH}}{C} \quad \frac{\epsilon_{λ₁}\text{pH}}{C} \quad \frac{\epsilon_{λ₁}\text{pH}}{C} \quad \frac{\epsilon_{λ₂}\text{pH}}{C} \quad \frac{\epsilon_{λ₁}\text{pH}}{C} \quad \frac{\epsilon_{λ₂}\text{pH}}{C}
\]

(Eq. 8)

Thus, because the l and C terms cancel out, the absorbance ratio in Eq. 8 depends only on pH. By choosing two wavelengths in such a way that \(\epsilon_{λ₁}\text{pH}\)/\(\epsilon_{λ₂}\text{pH}\) varies considerably with pH, one can obtain sensitive pH measurements. Experimenters using fluorescein derivatives typically use the peak absorbance wavelength (∼510 nm), and the isosbestic wavelength (∼440 nm), where ε is insensitive to pH changes. Monitoring dye absorbance at the isosbestic wavelength is attractive because one can determine the extent of dye loss during the experiment, assuming l is constant.

An advantage of absorbance for measuring pH is that it tends to be extremely stable and sensitive. On the other hand, because absorbance is proportional to l and C, a relatively high intracellular dye concentration is required, even for thick preparations (e.g., renal tubules).

**Fluorescence**

After absorbing a photon, most molecules return to the ground state by gradually losing energy through a series of random collisions with other molecules (46). Some dyes, however, can lose a quantum of energy from an excited singlet state by emitting a photon (i.e., fluorescing). The intensity of emitted fluorescent light (Iᵣ) can be measured with a photomultiplier tube (e.g., see ref. [75]) or an intensified
television camera, which provides imaging data (e.g., see ref. [412]). At most wavelengths, \( I_{\text{em}} \) is sensitive to pH, but at all wavelengths, \( I_{\text{ex}} \) is sensitive to dye concentration as well as other parameters (e.g., position of cell in incident light beam). Therefore, one usually uses a ratio technique to generate a parameter more uniquely related to pH. With the fluorescence-excitation ratio approach, commonly used with fluorescein dyes such as bis-carboxylethyl-carboxyfluorescein (BCECF), one alternately excites at two wavelengths while monitoring \( I_{\text{em}} \) simultaneously at one wavelength. With the fluorescence-emission ratio approach, commonly used with some rhodamine dyes such as seminaphthorhodafluor-1 (SNARF-1), one excites at one wavelength, while monitoring \( I_{\text{em}} \) simultaneously at two wavelengths. By analogy with the absorbance ratio approach, one chooses three wavelengths to optimize the pH sensitivity of the ratio.

Fluorescence measurements offer the advantage of being extremely sensitive. Thus, it is possible to quantitate the fluorescence from small amounts of dye in a portion of a single cell. In addition, one can use fluorescence with two-dimensional imaging, confocal microscopy, and two-photon microscopy. Because fluorescence is more sensitive than absorbance to the environment of the dye molecule, fluorescence measurements are in principle more prone to artifact.

**Calibration**

**SIMULTANEOUS MICROELECTRODE MEASUREMENTS**

Because dyes interact with cytoplasmic components, the spectroscopic properties of an intracellular dye differ, sometimes markedly, from those of the same dye examined in a cuvette. Therefore, intracellular dye calibration is essential. One calibration approach is to use a second, independent method for measuring pH, in the same cell or another cell under similar conditions. For example, simultaneous measurements with a pH-sensitive microelectrode have confirmed that the absorbance indicator dimethylcarboxyfluorescein in salamander proximal-tubule cells (107), and the fluorescence indicator BCECF in leech glial astrocytes (395) yield reasonable values.

**NIGERICIN APPROACH**

The most popular approach has been to monitor intracellular absorbance or fluorescence while using the high-[K\(^+\)]/nigericin technique (542) to clamp pH, to predetermined values. Nigericin is a carboxylic ionophore that exchanges K\(^+\) (and to a lesser extent Na\(^+\)) for H\(^+\) across cell membranes. If one is successful in choosing [K\(^+\)]\(_o\) to match [K\(^+\)]\(_i\), then pH\(_i\) should equal pH\(_o\). Thus, by altering pH\(_o\), one can measure the dye’s spectral properties over a range of pH values. A detailed calibration (i.e., spanning a wide pH range) can be obtained for each experiment. Alternately, one can perform the detailed calibration on one set of cells, and routinely perform only a single-point calibration (75). Potential problems with the high-[K\(^+\)]/nigericin technique have been discussed in some detail (106).

In using nigericin-containing solutions for dye calibration, one must be careful to cleanse the perfusion system completely after each calibration procedure. Even trace amounts of nigericin can interfere with the assessment of pH-regulating mechanisms, by mimicking a K-H exchanger and by increasing “background acid loading” through nigericin-mediated exchange of internal K\(^+\) for external H\(^+\) (49, 446).

**NULL-POINT APPROACH**

A novel calibration technique, originally proposed for microelectrodes by Szatkowski and Thomas (537), but applied to dyes by Eisner et al. (168), involves sequentially exposing the cell to a permeant weak acid and weak base. As discussed in subsequent sections, the size of the pH\(_i\) decrease caused by a weak acid increases predictably as the initial pH\(_i\) rises, whereas the size of pH\(_i\) increase elicited by a weak base decreases predictably as the initial pH\(_i\) rises. It is thus possible to compute the initial pH\(_i\) from the magnitude of the change of the pH\(_i\) indicator, assuming that the behavior of the weak acid and base is ideal and intracellular buffering power is fixed over the observed pH\(_i\) range. The application of this approach to fluorescent dyes is straightforward when pH\(_i\) is near the pK of the dye (i.e., when the pH dependence of fluorescence is approximately linear). In principle, the approach could be extended to more extreme pH\(_i\) values, although the mathematics would be more complicated.

Eisner et al. (168) have extended the approach by introducing an elegant null-point technique in which one experimentally determines a combination of weak-acid and weak-base concentrations that produces no change in the measured fluorescence. The null-point technique has the advantage of obviating the need for nigericin, but the disadvantage is that one must expose the cell to many solutions before zeroing in on the null point. Another limitation is that the dye is calibrated at exactly one pH\(_i\). Nevertheless, the null-point approach can be particularly useful in assessing the validity of other dye-calibration procedures, particularly the high-[K\(^+\)]/nigericin technique (77–79).

**Bleaching and Photodynamic Damage**

Excessive illumination can photolyse (i.e., bleach) dye molecules, causing a progressive decrease in the concentration of native dye. Among the photolysis products may be free radicals that react with cellular components and injure the cell ("photodynamic damage"). Excessive illumination can also cause a time-dependent shift in the apparent intracellular calibration curve of the dye (75), presumably due to bleaching-induced generation of dye-related products with spectral characteristics slightly different from those of the parent compound.

Bleaching can be particularly problematic during experiments on single cells, where the number of dye molecules

\[\text{We define "acid loading" as any process that causes pH}_i \text{ to fall. Examples include the uptake of H}^+\text{, the loss of HCO}_3^-\text{, or the metabolic production of H}^+.\]
and thus the number of emitted photons is low. The problem is that the limiting signal-to-noise (S/N) ratio is proportional to the square root of the number of photons measured. Although one can attempt to increase the number of emitted photons by increasing the intensity of the excitation light source, the greater intensity exacerbates the bleaching and photodynamic damage. These effects can be minimized by illuminating continuously with low-intensity light while integrating the signal over a sufficiently long period or by limiting the duty cycle of high-intensity exciting light (e.g., illuminate for 200 milliseconds every 2 seconds; 75). Thus, one must sometimes trade off pH resolution (i.e., S/N ratio) against time resolution. In imaging experiments, where a cell may be represented by thousands of picture elements (pixels), the photon emission rate per pixel is exceedingly low. Thus, one must trade off pHi resolution, time resolution, and spatial resolution (i.e., the number of pixels that must be grouped to compute pHi values).

Two-photon excitation laser scanning microscopy is a relatively new imaging technique that has allowed investigators to reduce photobleaching of ion-sensitive fluorescent dyes, even while acquiring high spatial and temporal resolution recordings (153, 535). Multiphoton imaging technology is based on the general quantum principle that a molecule can exhibit fluorescence after absorbing two photons simultaneously when excited by high-intensity light of twice the wavelength necessary for single-photon absorption. Because two-photon absorption and subsequent fluorescence rise very steeply with excitation intensity, lower intensity out-of-focus light does not generate appreciable fluorescence. In addition, excitation at the higher wavelengths (and thus lower energies) reduces overall photobleaching of the dye. Thus, two-photon imaging can be used to perform high-resolution imaging without the drawback of excessive photodynamic damage. As reviewed by Dunn and Young (165) and Zipfel et al. (620), two-photon microscopy has been particularly useful in examining intracellular Ca\(^{2+}\) transients, especially in cellular microdomains such as dendritic spines. However, the technique has also been used with BCECF to measure intracellular pH in microdomains of the epidermis (244).

Choice of Dyes

**BCECF**

The most popular dye for fluorescence measurements of pH is the fluorescein derivative BCECF (449), which has four negatively charged carboxylate groups and a phenolic =OH moiety that is titrated by pH changes. The dye can be directly loaded into large cells (e.g., *Xenopus* oocytes) with an injection pipette or into small mammalian cells by diffusion from a patch pipette during whole-cell recordings (344, 553). However, BCECF is usually loaded into cells as an uncharged tetra-acetoxyxymethylester (AM) precursor that easily permeates most plasma membranes. Intracellular esterases hydrolyze BCECF-AM to yield four or five formaldehyde molecules for each charged BCECF molecule trapped inside the cell. Dye loading can vary greatly among cell types—some take up large amounts of the precursor and convert it to BCECF very rapidly (i.e., ~1 minute), whereas others may not load sufficiently even after many minutes. Less conventional methods for dye loading, including scrape loading, osmotic lysis, and electroporation, are examined in more detail in Giuliano and Taylor (216). BCPCF, with carboxypropyl groups, is a derivative of BCECF that can be used as a dual-emission pH indicator (336).

**OTHER DYES**

Pyranine-based (215) and rhodamine-based dyes (557, 592) have been used for monitoring pHi. Rhodamine dyes such as SNARF-1 are excited and emit at longer wavelengths than the fluorescein derivatives. An advantage of SNARF-1 is its absorbance and fluorescence excitation spectra are pH sensitive, as is its fluorescence-emission spectrum (i.e., it can be used in the dual-emission mode). The pH sensitivity of its fluorescence-emission spectrum makes SNARF-1 more useful than fluorescein derivatives for confocal microscopy, in which one typically excites at only one wavelength. Dual-emission dyes also enable the user to sample more frequently and avoid the delay in alternating between two excitation wavelengths. SNARF-1 can be used simultaneously with fura-2 for monitoring both pH\(_i\) and [Ca\(^{2+}\)]\(_i\) (356). In general however, one should be cautious in using multiple ion-sensitive dyes simultaneously to avoid quenching artifacts (357).

**MEASUREMENT OF pH IN ORGANELLES**

Investigators have developed ingenious methods for targeting pH-sensitive probes to organelles. As reviewed by Maxfield and Yamashiro (360), pH-sensitive indicators can be targeted to the endocytic pathway following pinocytosis or receptor-mediated endocytosis. More recently, Kim et al. (292) have measured Golgi pH with either rhodamine- or fluorescein-labeled β subunit of verotoxin, which accumulates in the Golgi complex after receptor-mediated endocytosis and retrograde transport. Grinstein’s group has also measured pH in the endoplasmic reticulum (291) and the trans–Golgi network (152) by creating chimeric proteins with organelle-specific retrieval signals and subsequently tagging them with pH-sensitive fluorophores before internalization. A similar approach has been used to measure the pH of recycling endosomes (134, 541). The Machen group has examined pH regulation in the secretory pathway by targeting biotin-labeled pH probes to organelles expressing avidin-chimera proteins (596–598). Seksek et al. (492) have used a different technique to measure the trans–Golgi pH of fibroblasts. They injected the cells with 70-nm liposomes containing membrane-impermeable pH-sensitive fluorophores; the liposomes then fused with the trans–Golgi. As described in the next section, investigators have also measured the pH of organelles by fusing pH-sensitive green fluorescent protein (GFP) variants to protein markers for specific organelles.
GREEN-FLUORESCENT PROTEIN

Another fluorophore that has become useful in the pH field is GFP, which is a natural product of the jellyfish *Aequorea victoria*, and typically used to label proteins expressed in cells (108, 353). Several researchers have engineered GFP mutants such as pHluorins (366) that are sensitive to pH changes in the physiological range and can be targeted to the cytosol or organelles (159, 272, 309, 340, 346, 366, 388, 450). Compared with more traditional pH indicators, pH-sensitive GFP mutants are advantageous in displaying a low rate of photobleaching while remaining trapped inside of cells. pHluorins linked to markers of synaptic vesicles (synaptophylohuorins) undergo marked changes in fluorescence upon exocytosis at synaptic terminals and have therefore been used to characterize synaptic vesicle cycling associated with presynaptic activity (366, 474, 610). More recently, such vesicular cycling has been examined in transgenic mice expressing synaptophylohuorin (333). It is intriguing to speculate on the potential applicability of pH-sensive GFPs to examine cellular pH physiology. For example, one could perform in vivo pH measurements on targeted cells that are induced by a specific promoter to express a pH-sensitive GFP.

Differential Dye Loading

Recall that some cells load more rapidly than others with dyes such as BCECF. One can actually exploit such differential dye loading when working with mixed populations of cells. For example, intercalated cells in the cortical collecting tubule of the kidney incorporate BCECF-AM much more rapidly than the neighboring principle cells (484). In a somewhat different application, Chu et al. (121) found that mouse colonic crypt cells exclude SNARF, which then can be used to measure extracellular pH changes in the intact epithelium. BCECF generally leaks out of cells slowly because the dye is negatively charged. However, a probenecid-sensitive organic-anion transporter can extrude BCECF from thyroid cells (210) and epithelial kidney and intestinal cells (13, 125). In an elegant study, Harris et al. (247) used BCECF to measure the pH of the lateral intracellular space (LIS) of MDCK cell monolayers by first loading the cells with BCECF and subsequently allowing organic transporters to move the dye into the LIS.

BCECF generally stains the cytoplasm rather uniformly, although in some cells the nuclear region is more intense than peripheral areas (439). Working on Ehrlich ascites tumor cells, Thomas et al. (542) found that 6-carboxyfluorescein (when loaded into cells as the diacetate ester) is confined to the cytoplasm, but fluorescein is distributed in both the mitochondria and cytoplasm. In a more recent study, Slayman et al. (513) found that BCECF can accumulate in vacuoles of the fungus *Neurospora* when the cells are exposed to BCECF-AM.

One can evaluate a dye’s intracellular compartmentalization by monitoring the fluorescence loss elicited by selectively permeabilizing different compartments with detergents. For example, most of the BCECF loaded into rat hippocampal CA1 neurons appears to be in the cytoplasm because 0.01% saponin reduces the fluorescence signal by ~96% (48). Both absorbance- and fluorescence-derived estimates of pH, can be influenced by dye in compartments other than the cytoplasm, depending upon each compartment’s volume, dye concentration and pKₐ, and pH.

NUCLEAR MAGNETIC RESONANCE

Certain atomic nuclei, among them ³¹P and ¹⁹F, possess a quantum mechanical property termed "spin" and behave as tiny bar magnets with magnetic moments. When an atomic nucleus of this type is placed in an external magnetic field, the magnetic moment precesses with a characteristic frequency about the axis of the applied field. The nucleus can be excited to a high-energy state by irradiating it with an oscillating magnetic field of the same frequency (i.e., resonance frequency) as the precession frequency. The resonance frequency depends not only on the identity of the atomic nucleus (e.g., ³¹P), but also on its chemical environment, which influences the strength of the magnetic field at the nucleus. Thus, the resonance frequencies for ³¹P in HPO₄⁻ and H₃PO₄ are slightly different because of the different chemical environments of the ³¹P. Because the exchange rate of ³¹P between individual HPO₄⁻ and H₃PO₄ ions is very rapid, nuclear magnetic resonance (NMR) detects only a single inorganic phosphate peak, the location of which depends on [H₃PO₄]/[HPO₄⁻]. Because the dependence of this ratio on pH is described by a modified pH-titration curve (H₃PO₄ ⇌ HPO₄⁻ + H⁺; pKₐ = ~6.8), the position of the inorganic phosphate peak is a good index of pH.

A major advantage of ³¹P-NMR is that, in addition to providing nearly continuous measurements of pH, it can also be used to monitor levels of a variety of phosphorus-containing compounds, such as ATP. The pH value derived from NMR measurements is predominantly the pH of the cytoplasm as contaminated by inorganic-phosphate signals from other intracellular compartments, as well as the extracellular space. The mitochondrial inorganic phosphate peak can in some cases (503) be resolved from that of the cytoplasmic peak. NMR measurements have been made on whole organs, whole small animals, and human limbs (196).

A common variation of the NMR approach for monitoring pH, has been the use of ¹⁹F-labeled probes having pK values in the physiological range (156). Similar to ³¹P-NMR, ¹⁹F-NMR involves observing a chemical shift of the ¹⁹F-labeled probe in response to the level of protonation of an attached acidic group. As discussed by Deutsch (157), NMR measurements with ¹⁹F-labeled probes are advantageous over ³¹P in that background signals are low and the fluorinated probes are highly visible and sensitive to the environment. Some of the commonly used probes include fluoroanilines, derivatives of fluoroisobutyric acid, and fluorinated pyridoxins.
These probes are generally introduced into cells as methyl esters, similar to the approach used for pH-sensitive dyes. Aside from cost and the need for technical expertise, the major disadvantage of NMR is its relatively low sensitivity. Thus, a considerable mass of $^{31}$P or $^{3}$F is required for detection, precluding the use of the technique with single cells. For reviews, see refs. (196, 213, 402).

**FORCES AFFECTING THE PASSIVE MOVEMENT OF H$^{+}$ AND OTHER CHARGED ACIDS AND BASES**

**Forces Affecting H$^{+}$**

Until the 1930s, it was generally assumed that hydrogen ions were in electrochemical equilibrium across the cell membrane, as defined by the Nernst equation:

$$V_{m} = \frac{RT}{F} \ln \frac{[H^{+}]}{[H^{+}]}$$  \hspace{1cm} (Eq. 9)

Here, $V_{m}$ is the voltage difference across the plasma membrane (the unit is the volt), $R$ is the universal gas content (8.31 J × K$^{-1}$ × equivalent$^{-1}$), $T$ is absolute temperature, $F$ is Faraday’s constant (96,486 coulombs × equivalent$^{-1}$), and the subscripts $o$ and $i$ refer to extracellular and intracellular, respectively. In terms of pH, the forgoing equation becomes

$$V_{m} = (0.0585 \text{ V}) \times (\text{pH}_{o} - \text{pH}_{i})$$  \hspace{1cm} (Eq. 10)

assuming a temperature of 22°C. Thus, if H$^{+}$ were in equilibrium, then pH$_i$ would be one unit lower than pH$_o$ for each $-58.5$ mV of membrane potential. However, such a situation would present severe problems for the cell, inasmuch as $V_{m}$ changes would shift the equilibrium distribution of H$^{+}$, and thus alter pH$_i$ and pH$_o$-sensitive processes. It was not until the mid 1930s that Fenn and colleagues (179, 180), in experiments on frog skeletal muscle, demonstrated that pH$_i$ is higher than expected for H$^{+}$ to be in electrochemical equilibrium. The authors, in effect, estimated $V_{m}$ from the ratio $[K^{+}]/[K^{+}]$, and measured pH$_i$ using the weak-acid method.

Now both $V_{m}$ and pH$_i$ can be measured directly, and for nearly all cells studied, pH$_i$ is well above the equilibrium pH$_o$. In vertebrate skeletal muscle, for example, $V_{m}$ is $\sim-90$ mV and pH$_i$ is $\sim7.1$ (456). Given a pH$_o$ of 7.4, Eq. 10 predicts an equilibrium pH$_i$ of $\sim5.9$, far lower than the actual pH$_i$. Rather than calculating equilibrium pH$_i$, one could equally well compute equilibrium potential for H$^{+}$, that is, the membrane voltage required for H$^{+}$ to be in equilibrium across the membrane ($E_{H^{+}}$). In skeletal-muscle example introduced previously (pH$_i = 7.1$, pH$_o = 7.4$), the $E_{H^{+}}$ computed from Eq. 9 or Eq. 10 is $\sim-18$ mV. Because the actual $V_{m}$ ($\sim-90$ mV) is more negative than $E_{H^{+}}$, H$^{+}$ is drawn into the relatively negative cell, driven by an electrochemical gradient of $90 \rightarrow 18$ or 72 mV. Thus, there is a substantial electrochemical gradient (1.2 pH units or 72 mV) favoring the passive influx of H$^{+}$. If the cell membrane were permeable to H$^{+}$, then the resultant H$^{+}$ influx would represent a “chronic intracellular acid load” (i.e., an acid load imposed on the cell for an indefinite period) that would tend to lower pH$_i$.

In the previous discussion, we made no assumptions about the mechanism of the hypothetical influx of H$^{+}$. It had generally been thought that H$^{+}$ flux across the plasma membrane occurs via nonspecific pathways. As extensively reviewed by DeCoursey (145), voltage-activated H$^{+}$ currents were first described in snail neurons (550) and have subsequently been characterized in numerous other cells, including epithelial cells (including kidney cells), connective tissue, skeletal muscle, lymphocytes, macrophages, granulocytes, and microglia. These channels become active during depolarizing pulses and conduct outward H$^{+}$ currents in the direction predicted for passive H$^{+}$ movement in highly depolarized cells (e.g., when the $V_{m}$ of snail neurons is more positive than $\sim-10$ mV). The depolarization threshold for channel activation is lowered by increasing the pH$_o$-to-pH$_i$ gradient.

Proton channels are sensitive to Cd$^{2+}$ and Zn$^{2+}$ and other polyanal cathions. In rat alveolar epithelial cells, the H$^{+}$ channels are very selective for H$^{+}$. The permeability of the channel to H$^{+}$ is approximately seven orders of magnitude greater than the permeability to TMA (115). Although unitary H$^{+}$ channels have not been detected, noise analysis suggests that they are extremely small (4–10 fA) (89, 147). However, according to more recent analyses with improved S/N current measurements on inside-out patches of human eosinophils, the unitary conductance is larger: $\sim40$ fS at pH 6.5 and 140 fS at pH 5.5 (116). It is still difficult to distinguish these single-channel conductances from those estimated from electrogenic transporters and pumps (42, 89, 116, 146).

The role of this voltage-activated H$^{+}$ current has not been precisely defined. However, the current could serve as an elegant, although temporary, H$^{+}$-extrusion mechanism that does not require the direct input of energy. A strong depolarization of the cell (e.g., during an action potential) would not only activate the H$^{+}$ conductance, but also shift $V_{m}$ to values more positive than $E_{H^{+}}$, thereby reversing the electrochemical H$^{+}$ gradient. Increasing the pH$_o$-to-pH$_i$ gradient would also be expected to contribute to the activation of the H$^{+}$ conductance. Murphy et al. (385) demonstrated that Zn$^{2+}$-sensitive H$^{+}$ channels do contribute to the recovery of pH$_i$ from an NH$_3$-induced acid load in rat alveolar epithelial cells exposed to both normal and high-K$^{+}$ solutions. Finally, two groups—Ramsey et al. (437) and Sasaki and others (477)—have independently cloned a voltage-dependent, Zn$^{2+}$-sensitive H$^{+}$ channel.

**Forces Affecting Charged Weak Acids/Base**

In general, the passive flux of ionic weak acids (e.g., NH$_4$) and bases (e.g., HCO$_3$-) also impose a chronic intracellular acid load. As noted earlier (see Eq. 3), whenever a neutral weak acid (HA) is equilibrated across the cell membrane...
The transmembrane distribution ratio for $H^+$ is the reciprocal of the distribution ratio for the anionic conjugate weak base (A). Consider, for example, the CO$_3^-$/HCO$_3^-$ buffer system. If (1) $[CO_3^{2-}] = [CO_2]$ and (2) $CO_2$ is in equilibrium with $H^+$ and HCO$_3^-$ both inside and outside the cell, and (3) the equilibrium constant is the same inside and outside the cell, then

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[HCO_3^-]}{[HCO_3^-]} \quad (Eq. 11)$$

That is, the electrochemical gradient for HCO$_3^-$ (and that for any other monovalent anion that can be described by a similar equation) is equal to but opposite to the gradient for $H^+$. Inasmuch as $H^+$ normally leaks into cells, HCO$_3^-$ and other anionic weak bases tend to leak out, also decreasing pH. In crayfish muscle, the transmitter GABA opens Cl$^-$ channels that are also permeable to HCO$_3^-$ (280). The resultant HCO$_3^-$ efflux can reduce pH$_i$ by as much as 0.4. A similar GABA$_A$-activated HCO$_3^-$ conductance is present in cells from turtle cerebellum (111) and rat hippocampus (112, 279). This GABA$_A$-activated HCO$_3^-$ conductance can be inhibited by antagonists of GABA$_A$ receptors such as picrotoxin. Other anionic weak bases that seem to penetrate at least some cell membranes include the DMO anion (287), formate (494), propionate (286), and salicylate (494).

We can make a similar analysis for cationic weak acids ($HB^+ \rightleftharpoons H^++ B$). If the electrically neutral conjugate weak base (B) is equilibrated across the cell membrane (i.e., $[B]_i = [B]_o$), if the equilibrium $HB^+ \rightleftharpoons H^++ B$ holds on both sides of the membrane, and if the equilibrium constant is the same on both sides of the membrane, then

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[HB^+]_i}{[HB^+]_o} \quad (Eq. 12)$$

That is, the electrochemical gradient for $HB^+$ is in the same direction as that for $H^+$. Thus, similar to $H^+$, cationic weak acids such as NH$_3^+$ tend to enter the cell and produce a chronic intracellular acid load.

**Energetics of pH, Regulation**

The preceding analysis shows that, under the conditions that normally prevail in most cells (e.g., $pH_i \approx 7.1$, $V_m \approx -60$), the electrochemical gradients affecting $H^+$ and charged monovalent weak acids/bases (provided the neutral species equilibrates across the membrane) favor fluxes that would lower pH$_i$. These passive fluxes are depicted for a model cell in Fig. 2. Note that the above thermodynamic analysis addresses only the net direction of these passive fluxes, and does not address the rate of intracellular acid loading. How important for intracellular acid loading are these passive fluxes? To our knowledge, this issue has yet to be considered in a comprehensive fashion, probably for two reasons. First, one would like to be able to block specific individual pathways. Unfortunately, except for the examples of voltage-gated $H^+$ channels and GABA$_A$-activated HCO$_3^-$ conductances, no specific blockers of these pathways have been identified. Second, one would like to study these pathways in isolation. Unfortunately, cells may have multiple pathways for acid loading (e.g., cellular metabolism).

In predicting the passive flux of $H^+$ relative to that of monovalent acids/bases, one must consider factors other than the electrochemical gradients of the species involved. One such factor is concentration, which for $H^+$ may be several orders of magnitude lower than for ions such as HCO$_3^-$ or inorganic phosphate. Another key factor is membrane permeability, which for $H^+$ may be several orders of magnitude higher than for other ions (144). However, as described by DeCoursey (145) there are many difficulties associated with measuring membrane $H^+$ permeability.

The acid-loading mechanisms mentioned previously (i.e., passive fluxes, carrier-mediated transport, metabolism) are "chronic" because they act continuously to lower pH$_i$. Maintaining a normal pH$_i$ requires that acid loading be matched by a comparable—and continuous—extrusion of acid. By definition, this acid extrusion is an active (i.e., energy-requiring) process. Acid extrusion can be accomplished either by the active uptake of alkali (e.g., OH$^-$ or HCO$_3^-$) and/or the active removal of acid (e.g., $H^+$). As discussed in subsequent sections, a cell can also be subjected to an "acute intracellular acid load" (i.e., an acid load of defined and limited magnitude, imposed over a relatively

\[\text{We define "acid extrusion" as any process that causes pH} \rightarrow \text{rise. Examples include the efflux of } H^+ \text{ and the uptake of HCO}_3^-\text{.} \]
brief period of time). For example, injecting a cell with H+ produces a sudden decrease in pH. One of the key observations in pH physiology is that cells usually recover spontaneously from such acid loads by extruding the acid load.

Some metabolic products, which are commonly thought of as acids, or are indeed acids, may actually have no net effect on pH. A classic example is CO₂. In the steady state, the rate of cellular CO₂ production equals the rate at which CO₂ passively exits the cell, which equals the rate at which the CO₂ leaves the organism. Thus, because [CO₂] does not increase in the steady state, CO₂ production under these conditions is "pH silent." Another example of a pH silent metabolic product, at least in some cells, is lactic acid. For cells with an H+/lactate cotransporter that normally functions to transport lactate out of cells, lactic-acid production would be pH silent if the lactic-acid efflux via the cotransporter kept pace with lactic-acid production.

**EFFECTS OF WEAK ACIDS AND BASES ON pH**

**Effects of CO₂ and Other Neutral Weak Acids**

In the section, Distribution of Weak Acids and Bases, for measuring pH, we discussed the flux of neutral weak acids and bases across the cell membrane. In general, solutions containing the weak acid HA always contain the conjugate weak base A⁻. However, because membranes are usually far more permeable to HA than to A⁻, HA fluxes generally have a greater effect on pHi.

**FLUX OF THE NEUTRAL WEAK ACID**

When a cell is exposed to a neutral weak acid, HA enters and dissociates into A⁻ and H⁺, causing pHi to fall:

\[ HA \rightarrow A^- + H^+ \]  \hspace{1cm} (Eq. 13)

This process continues until [HA], equals [HAl]. Subsequently, there should be no further change in pHi, provided there is no flux of A⁻ and no change in the rates of acid-base transporter activity. Although CO₂ is often regarded as a weak acid, it is not an acid at all. Only after reacting with H₂O does CO₂ yield the true weak acid, H₂CO₃, which then dissociates (as described previously for the idealized weak acid HA):

\[ CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow HCO_3^- + H^+ \] \hspace{1cm} (Eq. 14)

These two reactions can be combined into a single one with an overall apparent equilibrium constant (Kₐ' = [HCO₃⁻][H⁺]/[CO₂]). The enzyme carbonic anhydrase (CA) greatly accelerates the formation of HCO₃⁻ from CO₂ by catalyzing the reaction:

\[ CO_2 + OH^- \rightarrow CA \rightarrow HCO_3^- \] \hspace{1cm} (Eq. 15)

which generates H⁺ by virtue of consuming OH⁻. The mechanism by which CA catalyzes this reaction is examined in more detail by Liljus et al. (334). In the absence of CA, the reaction can also occur, particularly under alkaline conditions (i.e., \( pH > 8 \)).

In the example of Fig. 3, a cell is successively exposed to solutions equilibrated with 1%, 2%, and 5% CO₂ (constant [HCO₃⁻] = 10 mM). Each time, CO₂ produces a rapid and sustained fall of pHi. The period during the CO₂ exposure in which pH is relatively stable is termed the "plateau phase". The magnitude of the CO₂-induced acidification is inversely related to the intracellular buffering power (B) (see following sections). The magnitude of the pH decrease also increases with [CO₂]o as does the fraction of incoming CO₂ that dissociates into HCO₃⁻ and H⁺ (see Eq. 14). The degree of dissociation is governed by the relationship between pHi and pKₐ', which, in logarithmic form, is the familiar Henderson-Hasselbalch equation:

\[ pH_i = pK'_a + \log \frac{[HCO_3^-]}{[CO_2]} \] \hspace{1cm} (Eq. 16)

where pKₐ' is ~6.1 at 37°C. Therefore, if pHi is 7.1, then 10 molecules of incoming CO₂ dissociate into H⁺ plus HCO₃⁻ for each molecule of CO₂ that remains CO₂. If the initial pHi is only 6.1, then this ratio falls to 1:1, and fewer incoming CO₂ molecules dissociate. There are two practical consequences of this rule. First, the lower the initial pHi, the smaller the magnitude of the acidification elicited by the subsequent exposure to the CO₂. Second, as one raises [CO₂]o by fixed increments, the magnitude of the acidification is not proportional to the successive [CO₂]o increment. Thus, in Fig. 3, the ΔpH produced by 2% CO₂ is less than twice as large as that produced by 1% CO₂, and that produced by 5% CO₂ is substantially less than five times that produced by 1% CO₂.

Although investigators have long considered that all gases penetrate all membranes simply by dissolving in the membrane lipid, Waibren et al. (573) demonstrated the first

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**FIGURE 3** Effect of CO₂ on pH. A giant barnacle muscle fiber was exposed to CO₂ at three different levels: 1% (pH = 7.5), 2% (pH = 7.2), and 5% (pH = 6.8). After its entry into the cell, CO₂ is rapidly hydrated to H₂CO₃, which in turn dissociates into H⁺ and HCO₃⁻, causing pH to fall. Removing CO₂ reverses these processes. pH was measured with an exposed-tip pH-sensitive microelectrode, of the design of Hinke. (Used with permission from Boron WF. Intracellular pH transients in giant barnacle muscle fibers. Am J Physiol 1977;233:C61–C73.)
membrane with negligible permeability to a dissolved gas (i.e., CO₂ and NH₃). Moreover, Nakhoul et al. (392) and Cooper and Boron (126) demonstrated the first gas channel, AQP1, which is permeable to CO₂. Studies indicate that AQP1 is responsible for ~60% of the CO₂ permeability of the human erythrocyte (170). AmrB, the bacterial Rh homolog, functions as an NH₃ channel (288, 615). Thus, at least two families of proteins can function as gas channels.

Weak acids such as acetic acid (389), lactic acid (504), and DMO (287) can also elicit intracellular acidifications, which have [HA]o and pH dependencies similar to those described for CO₂. CO₂ is unusual, however, in that [CO₃]o is easily controlled, independent of [HCO₃]o, either by the experimenter (who can equilibrate solutions with a known CO₂ mixture) or by the intact organism (which can alter its external respiration). Of course, the price one pays for varying [CO₂] at constant [HCO₃] is that—at equilibrium—pH must vary as well, as predicted by Eq. 16. In the laboratory, it is possible to overcome this limitation by using out-of-equilibrium CO₂/HCO₃ solutions (see following sections) to change only one of the three parameters (pH, CO₂, or HCO₃) at a time. For nonvolatile weak acids, the experimenter can only manipulate the total concentration of extracellular buffer ([TA]o = [HA]o + [A⁻]o) and pH. When [TA]o is fixed, changes in pH produce reciprocal alterations in [HA]o, and [A⁻]o, so that the magnitude of the HA-induced pH decrease is very pH sensitive.

**FLUX OF THE ANIONIC, CONJUGATE WEAK BASE**

In the preceding discussion, we assumed that the movement of the uncharged weak acid HA was the only factor affecting pH. In other words, we excluded the possibilities that: (1) the charged species A⁻ can traverse the cell membrane and significantly affect pH; and (2) acid-base transporters and metabolic processes that generate acid-base equivalents can alter the overall balance between acid extrusion/loading. In the absence of these complicating effects, applying and withdrawing HA/A⁻ would produce the pH changes illustrated in Fig. 4B.

What would happen if A⁻, as well as HA, were able to cross the membrane passively? At the instant a cell is exposed to a solution containing HA and A⁻, the electrochemical gradient for A⁻ would be inward (assuming that the initial [A⁻]o were sufficiently low). Initially then, both HA and A⁻ would enter, the HA tending to lower pHo, and the A⁻ tending to raise pH. A significant permeability of the membrane to A⁻ would reduce the initial rate of the HA-induced acidification. Eventually, however, the generation of A⁻ from incoming HA, as well as the influx of A⁻, would raise [A⁻]o, to such a level that A⁻ would tend to exit the cell passively, as described earlier. In addition, a transporter might move A⁻ out of the cell; for example, a Cl⁻+HCO₃ exchanger normally moves HCO₃ out of the cell. Subsequently, the efflux of A⁻ would lower pHo, both during the initial HA-induced acidification and during the later plateau phase, as illustrated in Fig. 4A. This A⁻ efflux represents what might be termed a “semi-chronic” acid load, inasmuch as the pH decrease would continue until [A⁻]o was so low that A⁻ was equilibrated across the membrane. Removing external HA/A⁻ would elicit a rapid rise in pHo due to the efflux of HA from the cell. Note, however, that pH would rise to a value less than the initial pH. The magnitude of the shortfall would be directly related to the net acid loading due to A⁻ efflux. The pH changes illustrated in Fig. 4A, presumably due to permeability of the membrane to the A⁻ form of a weak acid, have been observed in snail neurons exposed to salicylic acid (494).

Acid-extrusion mechanisms can also influence the pH changes of a cell exposed to HA/A⁻. In practice, the effects described in the following paragraphs are due to one or more acid-extruders that outstrip acid loaders. These transporters are described in more detail later, as are their effects on the dynamics of pH regulation. We introduce these transporters here only to complete our consideration of pH transients elicited by HA/A⁻. Consider a cell that is permeable to HA, but not to A⁻, and that has an acid-extrusion mechanism that is unaffected by A⁻ (e.g., Na⁺/H⁺ exchange). Applying HA/A⁻ would acutely acid load the cell, due to the influx of HA, and subsequent formation of A⁻ and H⁺. However, an acid-extruder stimulated by the pH decrease would remove this acute acid load and return pHo to exactly its starting value (Fig. 4C). Note that

![Figure 4](image-url)
removing HA/A\(^{-}\) causes pH\(_{i}\) to overshoot its initial value by a substantial amount. The magnitude of the overshoot is directly related to the net amount of acid extruded during the HA/A\(^{-}\) exposure. This pattern of pH\(_{i}\) changes, including a complete recovery of pH\(_{i}\) during the plateau phase, is observed in snail neurons acid loaded by exposure to CO\(_2/HCO_3^-\) (545). Other examples have been reported in which the plateau-phase pH\(_{i}\) recovery is incomplete.

As illustrated in Fig. 4D, exposing a cell to HA/A\(^{-}\) may cause the pH\(_{i}\) during the plateau phase to rise to a value even greater than the initial pH\(_{i}\). For example, if the cell had an acid extruder that is stimulated either by HA or A\(^{-}\), then the influx of HA would initially acidify the cell. However, the stimulated acid extruder would not merely return pH\(_{i}\) to its initial value, as in Fig. 4C, but increase pH\(_{i}\) beyond the initial value, as in Fig. 4D. We can provide two such examples in which A\(^{-}\) stimulates acid extrusion and thereby elicits pH\(_{i}\) changes similar to those in Fig. 4D: (1) exposing renal mesangial cells to CO\(_2/HCO_3^-\) stimulates a Na\(^{+}\)/H\(^{+}\) exchange driven HCO\(_3^-\) uptake mechanism (75, 76), and (2) adding acetic acid/acetate to the lumen of a rabbit S3 proximal tubule stimulates a Na\(^{+}\)/acetate cotransporter (389, 393). HA can also stimulate acid extrusion. In the rabbit proximal tubule, CO\(_2\) per se appears to stimulate Na-H exchange and H\(^{+}\) pumping (391).

**EFFECTS OF NH\(_3\) AND OTHER NEUTRAL WEAK BASES**

Solutions containing the neutral weak base B also contain its conjugate weak acid HB\(^{+}\). Because membranes are usually far more permeable to B than to HB\(^{+}\), B fluxes tend to have a greater effect on pH\(_{i}\).

**Flux of Neutral Weak Base**

When a cell is exposed to a neutral weak base, B enters and associates with H\(^{+}\) to form HB\(^{+}\), causing pH\(_{i}\) to rise:

\[
B + H^+ \rightarrow HB^+ \quad \text{(Eq. 17)}
\]

This process continues until [B], equals [B],. Subsequently, there should be no further change in pH\(_{i}\), provided there is no flux of HB\(^{+}\) and no change in the rates of acid-base transporter activity. Removing external B reverses the reaction as written in Eq. 17, and B passively diffuses out of the cell. pH\(_{i}\) should then return to exactly its initial level. An example of the effect of the weak base NH\(_3\) is shown in Fig. 5. Applying 20 mM total ammonium (i.e., [NH\(_3\)]\(_{o}\) + [NH\(_4^+\)]\(_{o}\)) causes pH\(_{i}\) first to rise, and then stabilize (segment ab, pulse 1). Removing the NH\(_3\)/NH\(_4^+\) elicits a fall in pH\(_{i}\) to a value somewhat lower than the initial one (compare points a and c). The reason for this small pH\(_{i}\) undershoot will become clear in the following sections. As discussed above for HA-induced acidifications, the magnitude of the NH\(_3\)-induced alkalization depends on intracellular buffering power, [NH\(_3\)]\(_{o}\), and the degree to which entering NH\(_3\) is protonated. The last is governed by the relation:

\[
\text{pH}_{i} = pK'_{a} + \log \left( \frac{[\text{NH}_3]}{[\text{NH}_4^+]} \right) \quad \text{(Eq. 18)}
\]

where pK\(_{a}'\) (-9.2 at 22°C) is the acid dissociation constant. Thus, at the initial pH\(_{i}\) of 7.3 (point a), approximately 99.4% of entering NH\(_3\) is protonated to form NH\(_4^+\), whereas at a pH\(_{i}\) of 7.8 (point b), only approximately 98% is protonated. The dependence of the protonation of NH\(_3\) on the difference (pK\(_{a}'\) - pH\(_{i}\)) has two consequences, analogous to the two discussed earlier for CO\(_2\). First, the higher the initial pH\(_{i}\), the smaller the magnitude of the alkalization elicited by the subsequent exposure to the NH\(_3\). Second, as one raises [NH\(_3\)]\(_{o}\), by fixed increments, the magnitude of the alkalization with each successive [NH\(_3\)]\(_{o}\) increment does not increase in proportion. Thus, in Fig. 5, the alkalization produced by 10 mM total NH\(_3\) is less than twice as great as that produced by 5 mM total NH\(_3\).

In Fig. 5, the magnitude of the NH\(_4^+\)-induced alkalization actually depends more on [NH\(_3\)]\(_{o}\) than on [total NH\(_3\)]\(_{o}\). Thus, the NH\(_4^+\)-induced alkalinations will be identical for solutions in which [total NH\(_3\)] and pH\(_{i}\) are varied reciprocally so as to keep [NH\(_3\)]\(_{o}\), constant (56). Neutral weak bases such as lidocaine (50), procaine (50), and methylamine (67) also elicit intracellular alkalinations that have [B], and pH\(_{i}\) dependencies similar to those described for NH\(_3\).
As noted previously, the bacterial protein AmtB, an Rh homologue, functions as a gas channel permeable to NH$_3$ (288, 386, 615). In addition, aquaporins-1, -4, -5, and -8 (387, 475) are permeable to NH$_3$.

**Flux of Cationic, Conjugate Weak Acid**

When one exposes a cell to a weak base, the dominant effect on pH$_i$ generally reflects the influx of the highly permeant neutral weak base (e.g., NH$_4$). However, the flux of the conjugate weak acid (e.g., NH$_3$) may produce pH$_i$ decreases that are substantial or even dominant. The effects on pH$_i$ of applying and withdrawing NH$_3$/NH$_4$ can be separated into four steps. When a cell is exposed to NH$_3$/NH$_4^+$, the rapid influx of NH$_3$ leads to an initial rise in pH$_i$ (Fig. 6A). However, this alkalinization is actually undershot, and outside the cell. pHi is transiently stable. During the plateau phase, the entry of NH$_4^+$ gives up its H$^+$ and exits the cell as NH$_3$. Because NH$_4^+$ had accumulated in the cell during the plateau (either because of NH$_3$ uptake per se or other acid-loading processes), pH$_i$ undershoots its initial value and the cell is acid loaded.

The acidifying effect of NH$_3$/NH$_4^+$ exposure lasts beyond the initial influx (Fig. 6A) and equilibration of NH$_3$ (Fig. 6B). During an extended plateau phase, the continuing net influx of NH$_4^+$ produces a plateau-phase acidification, as illustrated in Fig. 6C. In cells with a Cl$^-$-HCO$_3^-$ exchanger (e.g., sheep cardiac Purkinje fibers), this plateau-phase acidification may be augmented by efflux of HCO$_3^-$ in exchange for Cl$^-$ (566). When the external NH$_3$/NH$_4^+$ is eventually withdrawn, the pH$_i$ undershoot is greatly exaggerated (Fig. 6D). The magnitude of the undershoot reflects the previous influx of NH$_4^+$. The longer the exposure to NH$_3$/NH$_4^+$, the more extensive the plateau-phase acidification, and the larger the undershoot. This undershoot represents an acute intracellular acid load. Indeed, the NH$_3^-$/prepulse technique is widely used for acid loading cells in studies of pH$_i$ regulation (61).

If the NH$_3^+$ electrochemical gradient is reversed during the plateau phase, in a cell for which the dominant mechanism of NH$_3^+$ entry is a passive flux, then pH$_i$ rises during the plateau phase and the pH$_i$ undershoot is converted to a shortfall (56).

**FIGURE 6** The NH$_3^+$-prepulse technique. A: When a cell is first exposed to a solution containing NH$_2$ and NH$_3$, there is a rapid increase in pH$_i$ due to the influx of NH$_3$. This rise in pH$_i$ is blunted if NH$_3$ can also enter the cell. B: Eventually, [NH$_3$], rises to the point where [NH$_3$] transiently equals [NH$_3$]$_i$. Here, NH$_3$ is in equilibrium with NH$_3$ and H$^+$, both inside and outside the cell. pH$_i$ is transiently stable. C: During the plateau phase, the entry of NH$_3$ leads to the formation of intracellular NH$_3$, which then exits the cell. This sets up a shuttling system for carrying H$^+$ into the cell. The NH$_3$ may enter via a simple conductive pathway or be transported by a carrier such as the Na$^+$-K$^+$ pump or Na$^+$-K$^+$-Cl$^-$ cotransporter. Other acid-loading processes (e.g., Cl$^-$-HCO$_3^-$ exchange or metabolism) could also contribute to the plateau-phase acidification. D: Upon removing external NH$_3$/NH$_4^+$, most of the intracellular NH$_3$ gives up its H$^+$ and exits the cell as NH$_3$. Because NH$_3$ had accumulated in the cell during the plateau (either because of NH$_3$ uptake per se or other acid-loading processes), pH$_i$ undershoots its initial value and the cell is acid loaded.
INTRACELLULAR BUFFERING

Role of Buffering in pH$_i$ Regulation

In the broadest sense, a pH buffer is any system that moderates the effects of an acid or alkali load by reversibly consuming or releasing H$^+$, respectively. As we shall see, buffering in the intracellular fluid (e.g., the cytosol) is considerably more complex than in the blood and extracellular fluids. One can determine the buffering power (β) of the extra- or intracellular fluid by applying an acute acid or alkali load, and measuring the resultant change in pH. β is defined as the amount of strong base (or strong acid) required to raise (or lower) pH by a given amount. Strictly speaking, the definition is given in differential notation. However, if the amount of base added (ΔB, given in mM) and the resultant pH increase (ΔpH) are sufficiently small, then β is approximated by:

$$\beta = \frac{\Delta B}{\Delta pH} \quad \text{(Eq. 19)}$$

This definition was originally proposed in slightly different form by Koppel and Spiro in 1914 (311, 455) and later modified to its present state independently by Michaelis (365) and Van Slyke (564). Note that β is always a positive number; for a strong acid, Δβ and ΔpH are both negative. β is usually reported in the unit mM per pH unit.

Among the several methods available for applying acid and alkali loads to the cytoplasm, the most direct is by injection of acid (363, 545) or alkali into the cell with a micropipette. In all cells that regulate their pH$_i$, such an acute acid load produces an abrupt fall in pH$_i$, followed by a recovery towards its initial value (Fig. 7). The extent of the abrupt fall in pH$_i$ (segment $ab$) is determined by the amount of acid injected, the intracellular buffering power, and the ability of pH$_i$-regulatory mechanisms to blunt the pH$_i$ decrease. As discussed in later sections, these pH$_i$-regulatory mechanisms can actively extrude acid from the cell and thereby return pH$_i$ to its initial value ($bf$). Figure 7 shows a model experiment in which these pH$_i$-regulatory mechanisms are allowed to function. If we were to calculate β blindly, then we would see that the apparent β depends critically on the time of the ΔpH$_i$ measurement following the acid load. For example, the apparent β is relatively low at point $b$ and rises to infinity at point $f$, where ΔpH$_i$ is zero.

An approach for avoiding this ambiguity is to define intracellular buffering power (β) under conditions in which we completely block pH$_i$ regulation. In this case, pH$_i$ would follow the trajectory indicated by $ag$ in Fig. 7, the observed ΔpH$_i$ at “infinite” time would be relatively large, and the computed β would be smaller than any of the possibilities described previously. There are three justifications for excluding acid-base transport processes from the definition of β:

1. As we have just seen, excluding cellular transport processes makes the calculated β time independent (provided we allow enough time for pH$_i$ to fall).
2. As we shall see later, transport of acid and/or base can generally be distinguished from other buffering mechanisms by applying transport inhibitors or performing ion substitutions in the extracellular fluid.
3. Generally, these transport processes are not fully reversible. The buffering mechanisms included in our definition of β fall into three categories: (1) weak acids and bases (i.e., chemical buffers), (2) biochemical reactions consuming or releasing H$^+$, and (3) transport of acids or bases across organellar membranes. These mechanisms, which will be discussed in more detail later, can be justifiably grouped because there is presently no practical basis for distinguishing among them. Furthermore, they proceed very rapidly compared to the time currently required to measure pH$_i$.

Buffering mechanisms abate pH$_i$ changes produced by acute acid and alkali loads that otherwise could damage the cell. However, these mechanisms cannot prevent a change in pH$_i$ only reduce its magnitude. Furthermore, buffering mechanisms cannot restore pH$_i$ to its initial value following an acid or alkali load. Such recoveries are brought about by transport of acid and/or base across the cell membrane. As an illustration, consider the response of the cell to an acute intracellular acid load, as in Fig. 7. Cellular buffers respond very rapidly, typically consuming more than 99.99% of the applied acid and limiting the initial fall in pH$_i$ ($ag$). Active transport mechanisms respond more slowly, extruding acid from the cell and returning pH$_i$ toward normal ($bf$).

During the transport-mediated pH$_i$ recovery, H$^+$ previously taken up by the buffering mechanisms is now released and extruded from the cell. By the time pH$_i$ returns to its initial level ($f$), the entire acid load has been extruded from the cell.
the cell, and cellular buffering mechanisms have been returned to their pre-acid-loading state (a). Buffering mechanisms play no role in the steady state; in this case, acid loading exactly balances acid extrusion, and the state of cellular buffers is unchanged.

**MECHANISMS OF INTRACELLULAR BUFFERING**

The total intracellular buffering power ($\beta^*$) is the sum of chemical (i.e., weak-acid/base equilibrium), biochemical, and organellar buffering powers.

**Chemical Buffering**

A weak base ($B$) of valence $n$ reacts with $H^+$ to produce its conjugate weak acid ($HB$) of valence $n+1$:

$$B^n + H^+ \rightarrow HB^{n+1} \quad (Eq. 20)$$

This equilibrium is described by the relation:

$$K'_n = \frac{[B^n][H^+]}{[HB^{n+1}]} \quad (Eq. 21)$$

where $K'_n$ is the apparent acid dissociation constant. This relation can also be expressed in logarithmic form, whereupon it takes the form of Eq. 18. Starting with the definition of $\beta$ (Eq. 19) and the statement of chemical equilibrium (Eq. 21), one can express $\beta$ as a function of $[H^+]$, $K'_n$, and total buffer concentration (311, 365).

$$\beta = \frac{2.3K'_n[H^+]}{(K'_n + [H^+])^2}[TB] \quad (Eq. 22)$$

where 2.3 approximates ln(10), and [TB] is the concentration of total buffer (i.e., $[TB] = [B^n] + [HB^{n+1}]$). By obtaining the derivative of $\beta$ with respect to $[H^+]$ (i.e., $d\beta/d[H^+]$) and setting this equal to zero, one can show that $\beta$ is maximal when $[H^+]$ equals $K'_n$ (311). This maximal buffering power ($\beta^{max}$) is simply:

$$\beta^{max} \equiv 0.58 [TB] \quad (Eq. 23)$$

Although the pH of maximal buffering power is in general unique to the buffer, all buffers have the same molar buffering power ($\beta^{max}/[TB]$), and same dependence of $\beta$ on the difference $pH - pK'_n$. If more than one buffer is present, then $\beta^*$ is simply the sum of the individual buffering powers, each computed from Eq. 22.

The foregoing equation describing $\beta$ was derived by taking the partial derivative of $[B^n]$ with respect to $pH$ at a fixed [TB]. That is, the analysis applies only to buffers in a closed system, in which [TB] is constant. Closed-system buffers include a solution of a nonvolatile buffer (e.g., inorganic phosphate) in a beaker, a solution of a volatile buffer (e.g., CO$_2$/HCO$_3^-$) in a capped syringe, and an impermanent buffer (e.g., a protein) in the cytoplasm of a cell. In such a closed system, $\beta$ for CO$_2$/HCO$_3^-$ would be rather low at normal pH values, because pH, (7.4) is far higher than the $pK'_n$ of 6.1. However, cells are generally not a closed system with respect to CO$_2$/HCO$_3^-$, Rather, CO$_2$ usually freely exchanges with the extracellular fluid (ECF) so that the total intracellular CO$_2$ (i.e., $[CO_2] + [HCO_3^-]$) can change appreciably during intracellular acid-base disturbances. For example, when $H^+$ is added to cytoplasm containing CO$_2$/HCO$_3^-$, $H^+$ combines with HCO$_3^-$ according to the reaction:

$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow H_2O + CO_2 \quad (Eq. 24)$$

The cytoplasmic CO$_2$ so formed is lost to the ECF. Thus, it is $[CO_2]$, that remains constant, while $[HCO_3^-]$ and [total CO$_2$] both fall. Because there is no buildup of CO$_2$ within the cell, the extent of the reaction in Eq. 24 is limited only by the availability of HCO$_3^-$. The amount of $H^+$ absorbed (i.e., buffered) by the CO$_2$/HCO$_3^-$ system exactly equals the amount of HCO$_3^-$ that disappears. That is,

$$\beta^{CO_2} = \frac{\Delta[HCO_3^-]}{\Delta pH} \quad (Eq. 25)$$

where $\beta^{CO_2}$ is the CO$_2$ buffering power. When this equation, in differential form, is combined with the Henderson-Hasselbalch equation, it can be shown that

$$\beta^{CO_2} = 2.3 [HCO_3^-] \quad (Eq. 26)$$

when $[CO_2]$ is held constant. At very high pH values, when the equilibrium $H^+ + CO_2 \leftrightarrow HCO_3^-$ cannot be ignored, the term 4.6.[CO$_2$] must be added to the right-hand side of Eq. 26. This equation describes the buffering power of CO$_2$/HCO$_3^-$ in an open system, such as a solution in a beaker equilibrated with gaseous CO$_2$. Because most cell membranes are highly permeable to CO$_2$, this gas rapidly equilibrates across membranes and stabilizes $CO_3^-$. Provided $CO_2$ is balanced, the ECF behaves as an infinite reservoir for CO$_2$. Thus, an isolated cell in vitro behaves as an open system for CO$_2$/HCO$_3^-$. Because the intact organism has mechanisms (e.g., alveolar ventilation in higher vertebrates) for stabilizing [CO$_2$], the ECF, CO$_2$/HCO$_3^-$ behaves as an open-system buffer in vivo in the extra- and intracellular spaces.

In an open system, the CO$_2$/HCO$_3^-$ buffer pair generally makes a substantial contribution to $\beta^*$. For example, at a pH of 7.1, the buffering power of all non-CO$_2$ buffers in rat renal mesangial cells is only about 10 mM (75), whereas the computed $\beta^{CO_2}$ is nearly 29 mM when the cell is equilibrated with 5% CO$_2$. Thus, $\beta^{CO_2}$ accounts for nearly 75% of the $\beta^*$ of 39 mM/pH unit.

It is essential to distinguish clearly between open- and closed-system buffers of a cell. The cell is a closed system for intracellular buffers that do not readily cross the cell membrane (e.g., inorganic phosphate and the imidazole groups of proteins). Thus, these buffers are influenced by the attributes of the cell (e.g., volume, temperature, and metabolic state), and not those of the extracellular fluid. These are termed...
“intrinsic buffers” (56). Biochemical and organellar buffering mechanisms (vide infra) are also intrinsic, and the total intrinsic buffering power (B\(^{i}\)) is the sum of biochemical, organellar, and intrinsic chemical buffering powers.

The cell is an open system when one member of the buffer pair readily crosses the cell membrane. The buffering power of such a buffer pair is very sensitive to extracellular conditions. As noted earlier, \(\beta_{\text{CO}_2}\) is proportional to \([\text{HCO}_3^-]\), which in turn is completely determined by pH\(_i\) and the extracellular \(P_{\text{CO}_2}\) (assuming that \(\text{CO}_2\) equilibrates across the cell membrane). The cell also behaves as an open system for buffers other than \(\text{CO}_2/\text{HCO}_3^-\). These are generally conjugate pairs of which one member is a small, neutral molecule. Examples include \(\text{NH}_3/\text{NH}_4^+\) and acetic acid/acetate. Regardless of whether the charged species is a cation or an anion, the intracellular buffering power of an open-system buffer pair is always \(2.3 \times [\text{charged species}]\), provided the neutral species is equilibrated across the cell membrane. Thus, the intracellular buffering power of such a buffer pair is sensitive not only to the total amount of the buffer in the ECF (i.e., \([\text{TB}]_o\)), but also to pH\(_i\). As with \(\text{CO}_2/\text{HCO}_3^-\), these other open-system buffers can sometimes be the dominant component of B\(^{i}\). For example, pH\(_i\) in squid axons is \(\sim 9\) mM (61). When these axons are exposed to a pH\(_i\) \(- 7.7\) \(\text{NH}_3/\text{NH}_4^+\) solution containing only 10 mM total ammonium, \([\text{NH}_4^+]\), rises to 10 mM. The computed \(\beta_{\text{NH}_3}\) is 23 mM, nearly 70% of B\(^{i}\). Because the buffering power of such open-system buffers is so sensitive to factors external to the cell (e.g., pH\(_i\)), these buffers are termed “extrinsic” (56). The total chemical buffering power of a cell is the sum of the buffering powers of the individual conjugate acid/base buffer pairs, be they intrinsic (closed system) or extrinsic (open system).

### Biochemical Buffering

Because certain biochemical reactions consume or release H\(^+\) and are pH\(_i\) sensitive, they can act as H\(^+\) buffers (456). Examples include hydrolysis of ATP (which releases H\(^+\)) and hydrolysis of phosphocreatine (which consumes H\(^+\)). During the Cori cycle, the liver converts lactic acid into glucose, whereas skeletal muscle breaks down the glucose to produce ATP and more lactic acid. Also, H\(^+\) buffering can arise when reactions induce a change in the pK\(_i\)'s of an ionizable group. A classic example is the buffering reactions of oxygenated versus deoxygenated hemoglobin.

Well-studied examples of biochemical buffering are reactions involving intermediary metabolites in rat brain (183). Cells that are acutely acid loaded with an increase of \(P_{\text{CO}_2}\) in the ECF respond by reducing intracellular levels of several acid metabolic intermediates (i.e., the acids of lactate, pyruvate, citrate, α-ketoglutarate, malate, glutamate, and aspartate), while raising those of glucose and glucose-6-phosphate. These observations are consistent with the hypothesis that reducing pH\(_i\) inhibits a step in the glycolytic pathway, possibly the phosphofructokinase reaction. Indeed, mouse muscle phosphofructokinase is markedly sensitive to pH changes (556); reducing in vitro pH from 7.2 to 7.1 produces more than a 90% inhibition. Rat brain cells respond in the opposite fashion to acute intracellular alkali loads. Increases in pH\(_i\) induced by lowering the \(P_{\text{CO}_2}\) in the ECF lead to increased levels of lactate and pyruvate (348). Thus, the biochemical machinery of these cells seems to respond appropriately to pH\(_i\) changes, consuming H\(^+\) in response to intracellular acid loads and releasing H\(^+\) in response to alkali loads. The extent to which such biochemical reactions contribute to overall buffering power can be computed from changes in steady-state metabolite levels, provided that such changes are reciprocally linked to the production or consumption of neutral and/or readily diffusible molecules. For example, the consumption of one citrate molecule removes three H\(^+\), whereas malate removes two, and pyruvate, one. From data on rat brain (508), the buffering power of biochemical reactions amounts to about half that provided by all non-CO\(_2\) physicochemical buffers. Biochemical buffering power can be defined and quantitated in a manner analogous to chemical (vide supra) and organellar (vide infra) buffering powers (see Eq. 19). The biochemical buffering reaction can be written as:

\[
R^n + q \text{H}^+ \rightleftharpoons P^{n+q} \tag{Eq. 27}
\]

where R is the reactant, P is the product, n is the valence, and q is the H\(^+\) stoichiometry \((q > 0)\). The biochemical buffering power is thus:

\[
\beta = \frac{\Delta[R^n]}{\Delta pH} \tag{Eq. 28}
\]

As noted previously, biochemical and organellar (vide infra) buffering mechanisms, in addition to closed-system chemical buffers, comprise the intrinsic buffers.

### Organellar Buffering

H\(^+\)-transport systems have been identified or implicated in many intracellular organelles (456, 459), including mitochondria (83, 181, 369–372, 513, 514), lysosomes (401), sarcoplasmic reticulum (398), Golgi network (152, 292), endosomes (134, 237, 253, 394, 563), chromaffin granules (240, 401), and zymogen granules (22). In addition, three of the cloned Na-H exchangers (NHE6, -7, and -9) are found in organellar membranes (see Chapter 54) where they may contribute to organellar pH regulation (for reviews of vesicular H\(^+\) pumps, see [185, 217]). It would be reasonable to expect decreases in cytoplasmic pH to stimulate H\(^+\) uptake and/or inhibit H\(^+\) extrusion by at least some organelles and increases in pH\(_i\) to produce the opposite effects. Such buffering would lead to a net transfer of H\(^+\) into these organelles following intracellular acid loads and a net movement of H\(^+\) into the cytoplasm following alkali loads. A transfer of acid or base across organellar membranes would constitute a de facto buffer mechanism for the bulk intracellular
fluid. Although the extent to which such hypothetical organelar buffering mechanisms contribute to $\beta^i$ is not established, it can be inferred from published data that changes in extracellular pH can produce at least small changes in the intraorganellar pH of mitochondria (503), lysosomes (401), and sarcoplasmic reticulum vesicles (398). Organelar buffering power can be defined and quantitated in a manner analogous to that for physicochemical and biochemical buffering (see Eq. 19). If the organelar buffering reaction is written:

$$\text{Organelle}() + H^+ \leftrightarrow \text{organelle}(H^+) \quad \text{(Eq. 29)}$$

where the parentheses refer to organelar contents. Organelar buffering power is thus:

$$\beta = \frac{\Delta[\text{organelle}()]}{\Delta pH} \quad \text{(Eq. 30)}$$

Organelar buffers, along with the biochemical and closed-system chemical buffers, comprise $\beta^i$.

**MEASUREMENT OF INTRACELLULAR BUFFERING POWER**

**Titration of Cell Homogenates**

The easiest method for estimating $\beta$ is to homogenize a tissue sample and titrate the homogenate. The slope of the pH titration curve is the buffering power of the homogenate. However, some disadvantages limit the accuracy of this technique. First, homogenization-induced changes in metabolism must be prevented, for example by quick-freezing the sample before homogenization (193), and then performing the titration at low temperature (193) or in the presence of an inhibitor such as fluoride (508). However, with metabolism blocked, it is unlikely that the contribution of biochemical buffering can be accurately assessed. In addition, reducing temperature generally increases pK,a values of chemical buffers, and thus modifies the relationship between $\beta$ and pH. Finally, homogenization may disrupt cellular organelles and thus obscure the organelar buffering contribution. It might be possible to assess the organelar contribution to the buffering of the cytoplasm by using new techniques to monitor pH changes inside organelles (see previous section).

**Microinjection**

The most straightforward approach for estimating $\beta$ in an intact cell is to microinject a known amount of acid or base into a cell and monitor the resultant change in pH. The microinjection can be achieved by iontophoresis (545) or pressure injection (363). With the iontophoresis technique, two electrodes, one filled with acid or base and the other with a neutral salt, are placed in a cell, and electric current is passed between them. From the amount and duration of current, and from an in vitro calibration of current versus ejected acid/base, one can compute how much acid or base entered the cell. The pressure-injection method (363) also requires that the cell be impaled with two micropipettes, one for pressure-injecting the Cl$^-$ salt of an acid (e.g., HCl), and the other for monitoring intracellular Cl$^-$ activity ($\Delta a$). From the rise in $a$ and the Cl$^-$ activity coefficient, one can calculate the amount of HCl injected per unit volume of cytoplasm. This technique enables one to compute the total $\beta$, (i.e., sum of physicochemical, biochemical, and organelar buffering) of the cell. For small neurons incubated in a CO2-free solution, the iontophoresis and pressure-injection methods give $\beta$ values of 10.8 mM/pH unit (545) and 10.3 mM/pH unit (363), respectively. Disadvantages of this direct in vitro titration approach include the necessity of using cells that (1) are large enough to withstand the microelectrode impalements and (2) have a sufficiently compact geometry to permit rapid equilibration of the injected acid or base throughout the cell.

**Weak-Acid and Base Methods**

An approach that, at least in principle, is applicable to cells of all shapes and sizes, is measurement of the pH change produced by exposing the cell to a neutral weak acid HA (HA $\Leftrightarrow$ H$^+$ + A$^-$), or weak base B (HB$^+$ $\Leftrightarrow$ H$^+$ + B). As discussed earlier, exposing a cell to a neutral weak acid leads to a decrease in pH, as HA enters the cell and partially dissociates into H$^+$ and A$^-$. The entry and dissociation halt once [HA] = [HA]o. The extent of the dissociation depends on the weak acid’s pK,a, as well as the initial pH. Virtually all of the released H$^+$ is consumed by intracellular buffers (Fig. 8A); the remaining fraction is responsible for the accompanying pH decrease. Although HA/A$^-$ is a buffer, it does not participate in buffering the acid load imposed by the intracellular dissociation of HA. Similarly, a B/BH$^+$ buffer system can not buffer changes in pH caused by the entry (or exit) of B. Because the dissociation of HA leads to the formation of one A$^-$ for each H$^+$, the magnitude of the intracellular acid load is $\Delta[A^-]$. This amount of added acid is the additive inverse of the amount of base added ($\Delta B$) in the preceding definition of $\beta$:

$$\beta = \frac{\Delta B}{\Delta pH} = \frac{-\Delta[A^-]}{\Delta pH} \quad \text{(Eq. 31)}$$

Note that $\beta$ is the non-HA/A$^-$ buffering power of the cell. It is the sum of chemical, biochemical, and organellar buffering. $\Delta pH$ is directly measured, and $\Delta[A^-]$ is calculated from final and initial values of pH, and [HA]o. In the simplest case, the initial [A$^-$] is zero, and final [A$^-$] is given by:

$$[A^-] = [HA] \cdot 10^{pH-a-pK} \quad \text{(Eq. 32)}$$
FIGURE 8 Measurement of intracellular buffering power by exposure to a neutral weak acid or base. A: When a cell is exposed to a neutral weak acid (HA), HA enters the cell, where some of it dissociates into H\(^+\) and A\(^-\). Virtually all of this H\(^+\) is taken up by buffers (M; valence = n) that are titrated to their conjugate weak acids (HM; valence = n + 1). Thus, for each A\(^-\) formed, almost exactly one H\(^+\) is consumed by non-HA/A- buffers. \(\beta = -\Delta[A^-]/\Delta pH\). B: When a cell is exposed to a weak base, B, the base enters the cell, where some of it combines with H\(^+\) to form HB\(^+\). Virtually all of this H\(^+\) is derived from buffers (HM; valence = n + 1) that are titrated to their conjugate weak bases (HM; valence = n). Thus, for each HB\(^+\) formed, almost exactly one H\(^+\) is released by non-B/HB\(^+\) buffers. \(\beta = \Delta[HB^+]/\Delta pH\).

assuming [HA] = [HA], = [HA]. A commonly used weak acid for measuring \(\beta\) is CO\(_2\), for which:

\[
\beta = \frac{\Delta B}{\Delta pH} = -\frac{\Delta[HCO_3^-]}{\Delta pH}, \quad (\text{Eq. 33})
\]

\(\beta\) is the non-CO\(_2\)/HCO\(_3\) or intrinsic buffering power, \(\beta^\text{CO}_2\). Once \(\beta^\text{CO}_2\) is known, the total intracellular buffering power (\(\beta^\text{tot}\)) can be obtained by computing the open-system CO\(_2\) buffering power:

\[
\beta^\text{tot} = \beta^\text{CO}_2 + \beta^\text{IC} \quad (\text{Eq. 34})
\]

where \(\beta^\text{CO}_2\), the CO\(_2\) buffering power, is given by 2.3 \times [HCO\(_3^-\)], as outlined previously.

There are as many as three major drawbacks of using the weak-acid method to measure \(\beta\). First, [HA], may not exactly equal [HA]. In rapidly metabolizing cells for instance, [CO\(_2\)] may exceed [CO\(_3\)]. Second, the pH\(_i\) decrease elicited by the entry of HA may stimulate acid-extrusion mechanisms (i.e., pH\(_i\)-regulating systems described in subsequent sections). Thus, the actual \(\Delta pH\), may be smaller than if all acid-base transport had been blocked (Fig. 7). By underestimating \(\Delta pH\), one will overestimate \(\beta\). This error should in principle be eliminated by blocking the pH\(_i\)-regulating systems (56). One can also minimize the error by extrapolating the pH\(_i\)-versus-time curve back to a point at which acid extrusion is judged to have had no effect on pH\(_i\) (8, 47, 49, 113). For example, in the hypothetical experiment of Fig. 7, one could extrapolate the pH\(_i\) recovery (i.e., bedef) back to the time of the acid load. A third potential drawback of the weak-acid method is that the weak acid could indirectly alter pH\(_i\) by modifying cellular metabolism. Thus, it is critical that the weak acid affect pH\(_i\) only by entering and dissociating into H\(^+\) and A\(^-\). Otherwise, any other effect on pH\(_i\) will lead to an error in the calculation of \(\beta\).

The intrinsic intracellular buffering power can also be determined by exposing a cell to a neutral weak base, B (Fig. 8B). As described in the preceding section, exposing a cell to B leads to a pH\(_i\) increase as most of the entering B combines with H\(^+\) to form HB\(^+\). The entry of B and the alkali loading of the cell continues until [B] = [B]. Almost all of the entering H\(^+\) that is converted to HB\(^+\) is derived from cellular buffers. The minute amount that comes from the pool of free H\(^+\) is responsible for the rise in pH\(_i\). Thus, the change in [HB\(^+\)] is equivalent to the amount of strong base added to the intracellular fluid:

\[
\beta = \frac{\Delta B}{\Delta pH} = \frac{\Delta[HB^+]}{\Delta pH} \quad (\text{Eq. 35})
\]

In the simplest case, the initial [HB\(^+\)], is zero, and the final [HB\(^+\)], is calculated from the statement of chemical equilibrium:

\[
[HB^+] = [B] \cdot 10^{(\text{pK}_\text{HB} - \text{pH})}, \quad (\text{Eq. 36})
\]

assuming that [B] = [B] = [B]. The most commonly used weak base is NH\(_3\):

\[
[\text{NH}_3] \text{is determined in a manner analogous to that outlined earlier for [HCO}_3^-]. \text{Note that \(\beta\) in this case is the non-NH}_3/\text{NH}_4^- buffering power (i.e., the buffering power of all buffers other than NH}_3/\text{NH}_4^-). \text{If the NH}_3\text{titration is performed in the absence of CO}_2/\text{HCO}_3^-, \text{then the measured \(\beta\) is \(\beta\). \text{If CO}_2/\text{HCO}_3^- is present, then the measured \(\beta\) is \(\beta\) + \(\beta\) \text{CO}_2 = \(\beta\). \text{An approach commonly used with NH}_3\text{is to compute \(\beta\) from the pH}_i\text{decrease caused by decreasing or withdrawing external NH}_3. \text{During such experiments, one usually blocks acid-extrusion mechanisms (e.g., by removing external Na}^+) \text{that would likely counteract the acidification. By reducing [NH}_3], \text{in a stepwise fashion (Fig. 9A), one can compute \(\beta\) as a function of pH}_i\text{(Fig. 9B), as has been done in mesangial cells (75) and gastric parietal cells (590). An alternate approach one can use with NH}_3\text{is to compute \(\beta\) from the pH}_i\text{increase that accompanies stepwise increases of external NH}_3\text{. (49).}

ACID-BASE TRANSPORT SYSTEMS

Acid-base transporters can be divided into two groups: acid loaders and acid extruders. Acid loaders move H\(^+\) into or base equivalents (e.g., OH\(^-\) or HCO\(_3^-\)) out of cells and generally contribute to the recovery of pH\(_i\) from acute intracellular alkali loads. In contrast, acid extruders move H\(^+\) out of or base equivalents into cells and generally contribute to the recovery of pH\(_i\) from acute intracellular acid loads. In the remainder of this section, we discuss major acid-base transporters in each of these two categories. For each transporter,
principle, such passive transport processes could be exploited for protecting the cell against alkaline loads. Nevertheless, such passive processes generally do not appear to have a substantial impact on pH. A notable exception discussed above is the GABA<sub>A</sub>-activated Cl<sup>-</sup> channel at the crayfish neuromuscular junction (280) and in cells from turtle cerebellum (111) and rat hippocampus (112, 279). This Cl<sup>-</sup> channel can conduct HCO<sub>3</sub> and thus mediate a substantial HCO<sub>3</sub><sup>-</sup> efflux that lowers pH. For the most part, however, major acid-loading transport pathways are HCO<sub>3</sub> transporters—the most common ones being Cl<sup>-</sup>–HCO<sub>3</sub> exchange and Na<sup>+</sup>/HCO<sub>3</sub> cotransport.

**Cl<sup>-</sup>–HCO<sub>3</sub> and Cl<sup>-</sup>–OH<sup>-</sup> Exchangers**

**GENERAL FUNCTION AND MOLECULAR IDENTITY**

Found in a wide variety of animal-cell membranes, Cl<sup>-</sup>–HCO<sub>3</sub> exchangers normally couple the influx of Cl<sup>-</sup> to the efflux of HCO<sub>3</sub> (Fig. 10A). Na<sup>+</sup>-independent Cl<sup>-</sup>–HCO<sub>3</sub> exchangers (to distinguish them from the Na<sup>+</sup>-dependent Cl<sup>-</sup>–HCO<sub>3</sub> exchangers discussed later) are thought to serve two major functions in nonepithelial cells: regulation of intracellular pH (pH<sub>i</sub>), and regulation of intracellular [Cl<sup>-</sup>]. The erythrocyte exchanger is known as the "band 3 protein" because of its position on sodium dodecyl sulfate (SDS)–polyacrylamide gels (91). Band 3 has also been termed...
“AE1” because it was the first “anion exchanger” to be cloned and sequenced (310). As described in greater detail in Chapter 53, the AE gene subfamily comprises three related genes AE1, AE2, and AE3. The AE gene subfamily is a branch of the bicarbonate-transporter (SLC4) family that includes the Na+-coupled HCO$_3^-$ transporter subfamily (see Chapter 52). Several members of the SLC26 family can also mediate Cl$^-$-HCO$_3^-$ exchange (384).

**INVOLVEMENT OF Cl$^-$ AND HCO$_3^$/OH$^-$/ENERGETICS**

Red-cell AE1 mediates electroneutral homo- or hetero-exchange of monovalent anions with a 1:1 stoichiometry, exhibiting a substrate preference of Cl$^-$ ~ HCO$_3^-$ ~ NO$_3^-$ > Br$^-$ > F$^-$ > I$^-$ > divalent oxyanions (31, 274). Sulfate is cotransported with H$^+$ (367). Monovalent anion exchange can be reasonably well modeled by a ping-pong kinetic scheme, thought to reflect the alternation of an anion binding site between one side and the other of the membrane (90, 192, 306, 443). Because the Cl$^-$ : HCO$_3^-$ stoichiometry is 1:1, the transporter is electroneutral. Thus, the direction of net transport is determined by the sum of the chemical gradients for Cl$^-$ (i.e., [Cl$^-$]/[Cl])$_i$ and HCO$_3^-$ (i.e., [HCO$_3^-$]/[HCO$_3^-$]$_3$). Because the inward Cl$^-$ gradient is generally greater than the inward HCO$_3^-$ gradient in cells other than erythrocytes, the former dominates, driving HCO$_3^-$ out of the cell. The transporter is easily reversed, however, by inverting the sum of the Cl$^-$ and HCO$_3^-$ gradients. Indeed, a classic pH$_i$ assay for Cl$^-$-HCO$_3^-$ exchange involves removing external Cl$^-$: this evokes a rapid pH$_i$ increase (105, 565). In some cell types, the transporter can also be reversed by lowering [HCO$_3^-$]$_3$, as would occur during severe intracellular acid loads. The exchanger operating in reverse then becomes an acid extruder, moving HCO$_3^-$ into the cell and contributing to the pH$_i$ recovery from the acid load.

Some Cl$^-$-HCO$_3^-$ exchangers have a very high affinity for HCO$_3^-$ and transport can take place with low levels of HCO$_3^-$ (~100 μM) in a nominally HCO$_3^-$-free solution. For example, removing external Cl$^-$ from the solution bathing rat osteoclasts can elicit a dramatic 4,4′-disothiocyanatostilbene-2,2′-disulfonate (DIDS)-sensitive pH$_i$ increase, even in the nominal absence of CO$_2$/HCO$_3^-$ (507). However, vigorously bubbling the solutions with 100% N$_2$ gas greatly reduces the pH$_i$ increase.

Sun et al. (534) reported the presence of a Cl$^-$-OH$^-$ exchanger (or H$^+$/Cl$^-$ cotransporter), which operates in parallel with a “conventional” Cl$^-$-HCO$_3^-$ exchanger, in guinea-pig ventricular myocytes. The putative Cl$^-$-OH$^-$ exchanger is Cl$^-$ dependent; it is activated by low pH$_i$ (534) and high pH$_i$ (329). The evidence that it is a Cl$^-$-OH$^-$ exchanger, rather than a Cl$^-$-HCO$_3^-$ exchanger is that it functions as an acid loader in the presence or absence of CO$_2$/HCO$_3^-$, and is insensitive to DIDS (534). Activating the exchanger by lowering pH$_i$ is unaffected by a CO$_2$-free, 100% N$_2$ atmosphere (328), thereby ruling out the involvement of the small levels of HCO$_3^-$ present in air-equilibrated solutions.

Based on experiments on rat-brain synaptosomes, Martinez-Zaguián et al. (354) have concluded that a H$^+$/Cl$^-$ cotransporter is responsible for (1) the pH$_i$ increase elicited by removing extracellular Cl$^-$, and (2) the faster pH$_i$ recovery from an acid load in the absence of extracellular Cl$^-$. However, it is generally difficult to distinguish H$^+$ transport in one direction from OH$^-$/and/or HCO$_3^-$ transport in the opposite direction.

**DEPENDENCE ON pH$_i$**

By analogy to acid extruders, which are often stimulated by decreases in pH$_i$, one might expect acid loaders such as Cl$^-$-HCO$_3^-$ exchange to be stimulated by increases in pH$_i$. Indeed, based on DIDS-sensitive $^{36}$Cl$^-$ fluxes in Vero and L-cells (404), pH$_i$ increases elicited by returning Cl$^-$ to Cl$^-$-depleted mesangial cells (76), and rates of pH$_i$ change as well as $^{36}$Cl$^-$ fluxes in lymphocytes (358), Cl$^-$-HCO$_3^-$ exchange activity in these cells is low but measurable at pH$_i$ values as low as 6.5, increases to somewhat higher levels in the physiological pH$_i$ range, and then rises very steeply as pH$_i$ increases above ~7.6. Caution is in order concerning the two methods used to study the pH$_i$ sensitivity of Cl$^-$-HCO$_3^-$ exchange. First, isotopic fluxes reflect only unidirectional movements, and thus cannot distinguish Cl$^-$-Cl$^-$ exchange from Cl$^-$-HCO$_3^-$ exchange unless both influxes and effluxes are determined. Second, fluxes derived from rates of pH$_i$ change depend heavily on computed values of βCO$_2$, which (like the computed fluxes) rise steeply with increasing values of pH$_i$.

In more recent expression studies, the widely distributed AE2 polypeptides also appear to exhibit greater activity at more alkaline pH$_i$ values (263, 275, 327). The pH$_i$ sensor is located somewhere within the C-terminal transmembrane domain (527, 612), and its apparent pK$_a$ is modulated by residues within the N-terminal cytoplasmic domain (317, 527, 528). Multiple histidines within the transmembrane domain contribute to AE2 activity, as well as pH$_i$ and pH$_o$ sensitivities (529).

**EFFECTS OF PHARMACOLOGICAL AGENTS**

The nonerythroid Cl$^-$-HCO$_3^-$ exchanger is blocked by stilbene derivatives such as DIDS and 4-acetamido-4′-disothiocyanatostilbene-2,2′-disulfonate (SITS) (105, 403), as well as the noncompetitive inhibitor niflumic acid (132, 307). Some of the more potent AE inhibitors include oxonol dyes at the nanomolar level (14, 305, 308, 434).

**Cl$^-$-Organic Anion Exchangers**

In addition to the transporters mediating a direct exchange of Cl$^-$ for HCO$_3^-$, as described previously, some transporters exchange Cl$^-$ for organic anions that are the conjugate bases of weak acids (Fig. 10B). Although members of the AE family of Cl$^-$-HCO$_3^-$ exchangers can exchange organic anions for Cl$^-$ (132a, 274a), investigators have described other Cl$^-$-base exchange...
activities that cannot be attributed to AE family members. Since the last edition of this book, investigators have identified 10 SLC26 family members that transport monovalent and divalent anions, including sulfate, formate, and oxalate, in addition to chloride, bicarbonate, and hydroxyl ions. SLC26 proteins exhibit a range of ion specificities and many individual proteins exhibit functional versatility by transporting at least three types of anions (384). These proteins likely contribute to the recycling of organic anions associated with NaCl reabsorption in the proximal tubule. A detailed examination of these gene families is presented in Chapter 53.

Electrogenic Na\(^+\)/HCO\(_3\) Cotransporters

**General Function and Molecular Identity**

Present in the basolateral membrane of several epithelia, including the renal proximal tubule, is an electrogenic Na\(^+\)/HCO\(_3\) cotransporter (59) that mediates the isodirectional flux of one Na\(^+\) and—in the simplest model—three HCO\(_3\) ions (523). In epithelia, the cotransporter normally mediates a net efflux of Na\(^+\) and HCO\(_3\) (see following sections), and therefore functions as an acid load (Fig. 10C). In the early proximal tubule, Na\(^+\)/HCO\(_3\) cotransport appears to be the major route of HCO\(_3\) efflux across the basolateral membrane. The transporter thus plays a key role in HCO\(_3\) reabsorption, and probably makes an important contribution to Na\(^+\) reabsorption as well.

Our understanding of Na/bicarbonate cotransporters (NBCs), and other bicarbonate transporters has expanded immensely over the past 10 years with the molecular identification of these proteins. As described in Chapter 52, 10 SLC4 genes encode members of the family of bicarbonate transporters including electrogenic and electroneutral NBCs, the Na-driven Cl–HCO\(_3\) exchanger (NDCBE), and the Na\(^+\)-independent anion exchange (AES). At the amino-acid level, the transporters share considerable homology to one another, and are therefore predicted to have similar membrane topologies.

**Involvement of Na\(^+\) and HCO\(_3\) Energetics**

In the salamander proximal tubule, the cotransporter has an absolute requirement for Na\(^+\) and HCO\(_3\) (59). The cotransporter in rabbit proximal convoluted tubules can still function even in the absence of added HCO\(_3\) (312), probably due to intracellular formation of HCO\(_3\) from metabolically-derived CO\(_2\). Removing external HCO\(_3\) elicits a rapid fall in pH, and [Na\(^+\)], and a near-instantaneous depolarization of the basolateral membrane. A similar pattern of changes is caused by removing external Na\(^+\). All effects are fully reversible. The DIDS-sensitive depolarization elicited by removing Na\(^+\), which makes it appear as though Na\(^+\) is moving as an anion, is practically diagnostic of electrogenic Na\(^+\)/HCO\(_3\) cotransport.

A 1:3 stoichiometry is accounted for by any of the schemes outlined in Fig. 10C (341). The model in which Na\(^+\) exits together with one CO\(_3\) and one HCO\(_3\) (Fig. 10C, model B) is supported by the observation that SO\(_3\) stimulates Na\(^+\) uptake by basolateral membrane vesicles (519), presumably by interacting at a CO\(_3\) site. However, more recent studies indicate that NBCe1—A—at least as expressed in Xenopus oocytes—does not interact with SO\(_3\) or HSO\(_3\) (224, 225). Grichthchenko and Boron (222) performed a more direct assessment of CO\(_3\) transport by using extracellular pH electrodes to measure NBCe1-induced changes in surface pH (pH\(_e\)) of *Xenopus* oocytes expressing both the transporter and extracellular-facing carbonic anhydrase (CAIV). Depolarization-induced activation of the NBC elicited an expected decrease in pH, as base equivalents were transported into the cell. However, applying a CA inhibitor caused the magnitude of the pH, decrease to increase rather than to diminish. The larger response is consistent with the hypothesis that NBCe1 mediates CO\(_3\) transport and that the role of CAIV is to consume H\(^+\) generated as the reaction HCO\(_3\) → CO\(_3\) + H\(^+\) replenishes CO\(_3\) near the cell surface.

The direction of net cotransport is determined by the combined transmembrane gradients for Na\(^+\), HCO\(_3\), and voltage. If one focuses on Na\(^+\), then the epithelial cotransporter acts as an auxiliary Na\(^+\) pump. As far as its classification as a primary, secondary, or tertiary active Na\(^+\) transporter is concerned, the cotransporter occupies an unusual niche. The Na\(^+\)-K\(^+\) pump directly or indirectly establishes the membrane voltage. This pump also indirectly establishes the Na\(^+\) gradient that energizes the Na\(^+\)-H\(^+\) exchanger, which raises pH, and thereby establishes the HCO\(_3\) gradient. These voltages and HCO\(_3\) gradients, directly or indirectly established by the Na\(^+\)-K\(^+\) pump, drive the epithelial Na\(^+\)/HCO\(_3\) cotransporter in the direction of net Na\(^+\) efflux. Thus, this Na\(^+\) extrusion can be viewed as a secondary/tertiary active transport process.

**Effects of Pharmacological Agents**

Similar to Cl–/HCO\(_3\) exchangers, Na\(^+\)/HCO\(_3\) cotransporters are inhibited by stilbene derivatives such as DIDS (59, 454). Cloned NBCe1 expressed in oocytes can also be inhibited by benzamil (162), the nonsteroidal anti-inflammatory drug tenidap (162, 342), niflumic acid (337), and diBAC oxonol dyes (337).

Electroneutral K\(^+\)/HCO\(_3\) Cotransporter

**General Function and Molecular Identity**

A K\(^+\)/HCO\(_3\) cotransporter (Fig. 10D), presumably electroneutral, has been documented in squid giant axons, where it normally mediates the efflux of one K\(^+\) and one HCO\(_3\) ion (256). Because the transporter moves HCO\(_3\) out of the axon, it functions as an acid loader. A similar transporter has been reported in cells of the medullary thick ascending limb of rat kidney (331, 580) and may exist in cultured cortical astrocytes of rat (M.O.B. and W.F.B., unpublished data). In mammalian glia, K\(^+\)/HCO\(_3\) cotransport may contribute to the pH, decrease following an acute intracellular alkali load.
**Involvement of K^+ and HCO_3^-: Energetics**

In the squid giant axon dialyzed with a fluid containing K^+, but lacking Na^+ and Cl^-, the cotransporter moves K^+ and HCO_3^- out of the axon, eliciting a pH decrease (256). The base efflux is reduced by removing K^+ from the dialysis fluid. Also, the base efflux is reduced by elevating extracellular K^+ and HCO_3^-, thereby eliminating the gradient favoring K^+/HCO_3^- efflux. Under the appropriate conditions, the K^+/HCO_3^- cotransporter can also operate in the forward direction, moving HCO_3^- into the squid giant axon (142, 256). Exposing axons dialyzed with a fluid devoid of K^+, Na^+, and Cl^- to an artificial seawater containing K^+ and CO_2/HCO_3^- elicits a pH decrease (due to CO_2 entry), followed by a pH increase (due to K^+/HCO_3^- influx). The pH increase requires the presence of both extracellular K^+ and HCO_3^-.

The HCO_3^- fluxes mediated by the K^+/HCO_3^- cotransporter can be enhanced by using out-of-equilibrium CO_2/HCO_3^- solutions to introduce HCO_3^- exclusively to either the inside or outside of the axon (142, 614).

As mentioned previously, two independent groups have also reported K^+-coupled HCO_3^- transport in the medullary thick ascending limb (mTAL) from tubule suspensions (331) or perfused tubules (580). In both preparations, the K^+-coupled HCO_3^- transport did not require the presence of Na^+ or Cl^- and could be inhibited by DIDS. In the perfused-tubule study (580), luminal DIDS increased transepithelial HCO_3^- reabsorption—a finding consistent with DIDS-sensitive K^+-coupled HCO_3^- transport opposing HCO_3^- reabsorption.

**Effects of Pharmacological Agents**

While the transporter in the mTAL is sensitive to stilbene derivatives such as DIDS, the K^+/HCO_3^- cotransporter in the squid giant axon is not. However, quaternary ammonium ions introduced into the cell inhibit inwardly directed transport (142). The potency of inhibition increases with increasing alkyl chain length of the quaternary ammonium ion. For example, the apparent K_i for phenylpropyltetraethylammonium or PP-TEA (~91 μM) is ~850-fold less than the apparent K_i for tetraethylammonium or TEA (~78 mM).

**Ca^{2+}-2H^+ Exchange Pump**

**General Function and Molecular Identity**

The P-type ATPase family includes not only the Na-K and H-K pumps, but also the Ca^{2+} pumps, of which there are two types—the sarcoplasmic (endoplasmic) reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+}-ATPase (PMCA). These Ca^{2+} pumps, along with Na^+/Ca^{2+} exchangers, play a pivotal role in returning [Ca^{2+}], to low levels following stimuli-induced influx through Ca^{2+} channels in the plasma membrane or release from intracellular stores. SERCAs pump Ca^{2+} back into intracellular stores, whereas PMCAs pump Ca^{2+} out of the cell across the plasma membrane. In both cases, the pumps exchange Ca^{2+} for H^+. Thus, from the standpoint of pH, the PMCAs, which take up H^+ as they extrude Ca^{2+} across the plasma membrane, function as acid loaders (Fig. 10E).

Four PMCAs (PMCA1-4) have been cloned and characterized (see reviews by 100, 233, 375, 599). The cDNAs encode proteins with ~1200 amino acids. Based on hydrophy analysis, the PMCAs have 10 transmembrane α-helices (TM 1–10) with both the N and C termini being cytoplasmic. The ATPase catalytic domain lies between TM4 and TM5. Based on localization studies (see review [232]), PMCA1 and PMCA4 are expressed ubiquitously. In contrast, PMCA2 and PMCA3 are found predominantly in the nervous system. mRNA encoding all four isoforms have been found in kidney (102, 349). However, PMCA levels of mRNA and protein are highest in the distal tubule, particularly in the distal convoluted tubule (55, 350).

**Involvement of Ca^{2+}: Energetics**

It has been known for some time that the plasma-membrane Ca^{2+} pump in erythrocytes exchanges Ca^{2+} for H^+ (101, 396). Schwiening et al. (487) have used Ca^{2+} and pH-sensitive microelectrodes to demonstrate that the plasma membrane Ca^{2+} pump in snail neurons also exchanges extracellular H^+ for intracellular Ca^{2+}. In this preparation, activation of the Ca^{2+} pump (generated by increasing intracellular Ca^{2+} with depolarization or iontophoretic Ca^{2+} injection) elicits an increase in extracellular Ca^{2+}, and a simultaneous increase in intracellular pH. Both the increase in extracellular Ca^{2+} and pH can be inhibited by the Ca^{2+}-pump inhibitor vanadate. A similar Ca^{2+} pump, present in hippocampal neurons (554), appears responsible for causing an increase in the extracellular pH of the hippocampal slice elicited by stimuli-induced neuronal activity (408, 409, 518).

**Glutamate Transporters**

**General Function and Molecular Identity**

In the mammalian central nervous system (CNS), the predominant excitatory amino acids glutamate and aspartate are transported from the extracellular space back into neurons and glial cells by ion-coupled glutamate transporters in the SLC1 family (282). Since 1992, cDNAs encoding several of these electrogenic glutamate transporters have been cloned and characterized (27, 28, 135, 136, 172, 281, 284, 421, 531). These transporters are predominantly expressed in the nervous system, but also in certain epithelial cells (135, 453, 490). Because the nomenclature in this field is not uniform, we will follow the suggestion of Amara (28) and refer to them as excitatory amino acid transporters (EAATs). The L-glutamate/L-aspartate transporter (GLAST1 or EAAT1) and the GLT1 (or EAAT2) transporter are mainly found in glia such as astrocytes and Bergmann glia. In contrast, the excitatory amino acid carrier 1 (EAAC1 or EAAT3) and EAAT4 are found predominantly in neurons. EAAT3 is also expressed in liver and is also present in small intestine and kidney, where it mediates the uptake of glutamate and aspartate across the
apical membrane (250). Of the more recently cloned glutamate transporters, EAAT4 is expressed in cerebellum, whereas EAAT5 is predominantly expressed in retina.

**INVOLVEMENT OF GLUTAMATE/ASPARTATE, NA⁺, AND K⁺: ENERGETICS**

Using the results from 22Na⁺ and [14C]glutamate uptake studies and pH measurements on *Xenopus* oocytes expressing EAAT3, Kanai et al. (283) concluded that the protein transports one glutamate, two Na⁺ and one H⁺ into the cell, in exchange for one K⁺, which moves out of the cell (Fig. 10F). This stoichiometry, which predicts a net movement of one positive charge into the cell, agrees with that proposed for the glutamate transporter in salamander retinal glia based on kinetic analysis (70). Klöckner et al. (304) suggested that EAAT1, when expressed in *Xenopus* oocytes, has a stoichiometry of one glutamate and three Na⁺ into the cell, in exchange for one K⁺ out of the cell, without transport of an acid-base equivalent. However, in experiments on EAAT3-expressing oocytes, adding glutamate or aspartate to the extracellular fluid causes a steep fall in pHᵢ (283), consistent with the influx of H⁺ or the efflux of alkali.

**Peptide and Divalent-Cation Transporters**

Although most solute exchangers and cotransporters in animal cells are Na⁺-driven, some are H⁺-coupled (Figs. 10G and 10H). The absorption of oligopeptides and peptide-based antibiotics by intestinal and proximal-tubule epithelial cells is mediated by two members of the SLC15 family, H⁺/oligopeptide transporters PepT1 (SLC15A1) and PepT2 (SLC15A2) (see review [137, 197]). PepT1 and PepT2 transport the same di- and tripeptides, but with different affinities/capacities (137). PepT1 exhibits low-affinity, high-capacity transport, whereas PepT2 exhibits the opposite: high-affinity, low-capacity transport. Both transporters are expressed in kidney (among other tissues), with PepT1 found in the S1 segment of the proximal tubule and PepT2 found in the S3 segment (498, 516). More recently identified members of the peptide transporter family include PTH1 (SLC15A4) and PTH2 (SLC15A3), both of which can transport histidine as well as di- and tripeptides in a H⁺-coupled manner (137).

The cDNA encoding PepT1 has 707 amino acids with 12 putative membrane-spanning segments (175). In oocytes expressing PepT1, the net transport of dipeptides occurs with a proton: dipeptide stoichiometry of 1:1 (525). For negatively charged dipeptides, one H⁺ binds to the cation site of the transporter, whereas another H⁺ neutralizes the dipeptide on the peptide site. The net transport stoichiometry of negatively charged dipeptides is therefore one proton for one neutralized dipeptide. Adding extracellular glycyl-glycine, glycyl-leucine, or glycyl-lysine (dipeptides) to oocytes expressing PepT1 causes a steep fall in pHᵢ (283, 525), consistent with the H⁺ influx.

Gunshin et al. (235) cloned the cDNA encoding a mammalian divalent-cation transporter (DCT-1)—now called the divalent metal transporter (DMT)—that mediates intestinal nonheme Fe²⁺ absorption. DMT contains 561 amino acids with 12 putative membrane-spanning domains. By Northern blotting, DMT is ubiquitously expressed; heavily so in the duodenum. DMT is electrogenic, and cotransports any one of a group of metals (e.g., Fe²⁺, Zn²⁺, Mn²⁺, Co²⁺, Cd²⁺, Cu²⁺, Ni²⁺, Pb²⁺) together with one H⁺. By analogy with the EAATs and PepT1, adding Fe²⁺ to the extracellular fluid of an oocyte expressing DMT leads to a rapid fall in pHᵢ, consistent with the influx of H⁺.

**ACID-EXTRUSION MECHANISMS**

As discussed earlier, acid extrusion, that is, the movement of H⁺ (or protonated monovalent weak acids) out of cells, or the movement of OH⁻ or HCO₃⁻ (or monovalent weak bases) into cells, generally occurs against a steep energy gradient. Based on their source of energy, acid-extrusion mechanisms can be divided into three groups: primary, secondary, and tertiary active transporters (23). Primary active transport mechanisms, also called pumps, derive their energy from electron transport (e.g., the electron transport chain that drives H⁺ out of mitochondria) or, as with the transporters such as the H⁺ pump considered in the following sections, from the hydrolysis of ATP. Secondary active transport systems (e.g., Na⁺-/H⁺ exchange) derive their energy from the electrochemical gradient of an ion (e.g., Na⁺), a gradient that is in turn established by a primary active transporter (e.g., Na⁺-K⁺ pump). Finally, tertiary active transporters (e.g., the monocarboxylate system) are driven by an ion gradient established by a secondary active transporter.

From the point of view of feedback control, the effectiveness of an acid extruder would be enhanced greatly if decreases in pHᵢ increased transport. The most productive approach for studying the pHᵢ dependence of acid extruders in intact cells has been to load cells with acid acutely, as schematized in Fig. 11A. The acid load can be applied by pre-pulsing with NH₃ (61), microinjecting H⁺ (363), dialyzing with a low-pH solution (467), or reducing pHᵢ in the temporary presence of nigericin (228). In the model experiment of Fig. 11A, the pHᵢ decrease produced by the acid load (segment ab) is followed by a rapid recovery to the initial pHᵢ (bda). In the most general case, this pHᵢ recovery is mediated by one or more acid extruders, but may be opposed by one or more acid loaders. Identifying the component of pHᵢ recovery that is mediated by a particular transporter requires that the transporter be specifically, instantaneously and completely inhibited. Such inhibitors exist for two of the acid extruders that we will discuss, the Na⁺-driven Cl⁻/HCO₃⁻ exchanger and the Na⁺-/H⁺ exchanger. Figure 11A illustrates three possible outcomes (indicated by broken curves following the arrow) of applying such an agent during a pHᵢ,
recovery due at least in part to a mechanism sensitive to the blocker. First, applying the blocker may slow but not halt the pH recovery. This result indicates that a second, blocker-insensitive acid extruder also contributes to the pH recovery. Second, the blocker may completely eliminate the pH recovery, indicating that the entire recovery is due to a blocker-sensitive mechanism(s). However, complete blockade by the blocker does not rule out the possibility that the blocker-sensitive acid-extrusion process is opposed by a less potent blocker-sensitive acid-loading process. Third, applying the blocker may not only halt the pH recovery, but may also unmask a slow pH decline. This result implies that the blocker-sensitive acid-extrusion process is opposed by at least one blocker-insensitive acid loader.

In any of these three eventualities, the difference in rates of pH change (d pH/dt) immediately before and immediately after applying the blocker at point c in Fig. 11A is the component of the pH recovery—at the pH value prevailing at point c—that is due to blocker-sensitive transporters. In order to convert this blocker-sensitive d pH/dt to a net acid extrusion rate (JE), we must multiply this d pH/dt by the total intracellular buffering power (β) prevailing at the pH of the blocker application, as well as the inverse of the surface-to-volume ratio (ρ):

\[ J_E = (β^2/ρ) \times (d pH/dt)_{\text{blocker-sensitive}} \]  
(Eq. 38)

Because acid-base transporters are generally sensitive to changes in pH, this analysis must be repeated over a range of pH values to produce a plot of the pH dependence of the blocker-sensitive flux(es).

Rather than repeating the experiment in Fig. 11A many times, each with a different pH, we could use a more practical approach, which requires only three types of experiments. First, we monitor the pH recovery—in the absence of blocker—over the entire pH range indicated by bcd in Fig. 11B. At regular pH intervals during this recovery, we compute the “gross” d pH/dt, from which we compute the gross JE (Fig. 11C).
Second, we monitor the pHi recovery in an experiment in which the blocker is present during the entire pHi recovery. If the blocker-sensitive transporter is the only acid extruder present in the cell, then pHi will not recover at all, or may even decline. On the other hand, if other acid extruders are present (as is more often the case), pHi will recover slowly and incompletely, as in bc. At regular pHi intervals during this abbreviated recovery, we compute the “blocker-insensitive” \( \Delta pHi/dt \), from which we compute the blocker-insensitive \( J_b \) (Fig. 11C).

Third, we allow pHi to recover fully (bcd), and then add the blocker. If, during the steady state indicated by point d, one or more acid loaders oppose the blocker-sensitive acid extruder, as is typically the case, then applying the blocker will cause pHi to drift downward (de). The initial rate of pHi decline at point d reflects the rate of acid loading (\( J_b \)) at the pHi, at d. At regular pHi intervals during this pHi decline, we compute the “blocker-unmasked” \( \Delta pHi/dt \), from which we compute the blocker-unmasked \( J_b \) (Fig. 11C).

Finally, at each pHi value, we subtract the blocker-unmasked \( J_b \) from the blocker-insensitive \( J_b \) in Fig. 11D. The result is the pHi dependence of the “blocker-sensitive” \( J_b \) (i.e., the flux mediated by the transporter of interest). This approach was first used by Boron et al. (65) to determine the pHi dependence of the Na\(^+\)/H\(^+\) exchanger of renal mesangial cells. Others extended this approach to study the pHi dependence of Na\(^+\)/H\(^+\) exchange in IEC-6 colonic cells (512), UMR-106 osteoblastic cells (236), osteoclasts (439), rabbit S3 proximal tubules (113), 3T3 fibroblasts transformed with the c-H-ras oncogene (285), Chinese hamster lung fibroblasts (Yoon M.O.B. and W.F.B., unpublished data), rat hippocampal astrocytes (49) and shrunken mesangial cells (44). Finally, the approach outlined in Figs. 11B, 11C, and 11D also has been used to determine the pHi dependence of the Na\(^+\)/H\(^+\) driven Cl\(^-\)/HCO\(_3\)\(^-\) exchanger in NIH 3T3 cells transformed with the c-H-ras oncogene (285), and the pHi dependence of the H\(^+\) pump in rabbit S3 proximal tubules (113) and osteoclasts (439).

**H\(^+\)** Pumps

**GENERAL FUNCTION AND MOLECULAR IDENTITY**

An electrogenic vacuolar-type H\(^+\) pump (Fig. 12A, left) is present in the distal nephron of the kidney (185), as well as in macrophages and neutrophils (491, 536), osteoclasts (110), tumor cells (355), and goblet cells of the insect midgut (593). Similar H\(^+\) pumps are present in clathrin-coated vesicles and other organelar membranes (185, 186).

The V-type H\(^+\) pump is composed of two domains, V\(_0\) and V\(_1\). The V\(_0\) domain, involved in translocating H\(^+\) across the membrane, is a 260-kD complex of at least five different subunits (\(a, a, c, c^\prime, c^\prime\)), ranging from 17 to 100 kD. The H\(^+\) pump inhibitor DCCD binds to subunit c. The V\(_1\) domain, involved in ATP hydrolysis, is a 570-kD complex of at least eight different subunits (\(A-H\)), ranging from 14 to 70 kD. The catalytic nucleotide binding sites are located on subunit A. Also, the sulfhydryl reagent N-ethylmaleimide inhibits the pump by binding to subunit A. A more complete review of the structure and function of the V\(_0\) and V\(_1\) subunits of the pump is presented in refs. (187, 526). Finally, similar vacuolar H\(^+\) pumps have been examined in Neurospora and plant cells (72, 73, 619).

Immunohistological studies with antibodies raised against various subunits of the H\(^+\) pump have revealed expression of the pump throughout the nephron on both apical and basolateral membranes (571). In the cortical collecting tubule, the H\(^+\) pump is localized to the apical membrane of \(\alpha\) intercalated cells (which secrete acid) and the basolateral membranes of \(\beta\) intercalated cells (which secrete alkali) (84, 85, 509). Vacuolar H\(^+\) pumps are also present at the apical membrane of the proximal tubule (84, 85, 509).

**ENERGETICS**

The extrusion of H\(^+\) by this primary active transporter is not coupled directly to the movement of other ions that might balance electrical charge. Rather, this pump is electrogenic. Because the H\(^+\) pump is driven by the hydrolysis of ATP, which provides a substantial amount of energy, it is capable of generating a large H\(^+\) gradient across the cell.
membrane. For example, if the voltage across the membrane were zero, then the H⁺ pump could establish a pH difference of about three pH units. On the other hand, when compared with other H⁺ transporters, H⁺ pumps have a relatively low transport rate. Thus, the H⁺ pump is expressed in epithelia needing a high-gradient/low-capacity H⁺ transporter. A H⁺ pump contributes to pHi regulation in a number of cells, including macrophages and neutrophils (536), osteoclasts (439), renal cells (493, 572), glial cells (411, 420), and corneal epithelial cells (599).

**K⁺-H⁺ Exchange Pumps**

**GENERAL FUNCTION AND MOLECULAR IDENTITY**

As discussed earlier, K⁺-H⁺ exchange pumps (Fig. 12A, right) are members of the P-type ATPase family. Both the gastric and nongastric H⁺-K⁺ pumps are ATP-driven transporters that exchange intracellular H⁺ for extracellular K⁺. However, the gastric and nongastric pumps differ in their molecular biology, localization, functional properties, and pharmacological sensitivities (see reviews [270, 510]). Similar to the Na⁺-K⁺ pump, the H⁺-K⁺ pumps have a catalytic α subunit and a β subunit. The cDNAs encoding the two subunits of the gastric H⁺-K⁺ pump (502, 551) are similar to the corresponding subunits of the Na⁺-K⁺ pump (133, 176, 271, 374) revealing that they are ~65% homologous to the α subunits of the Na⁺ pump and the gastric H⁺-K⁺ pumps.

Based on localization studies, in some species, both the gastric and nongastric forms of the H⁺-K⁺ pump are present in the collecting duct of the kidney (see reviews [270, 510]). Although both forms are localized to the renal collecting duct, they are thought to play only a small role in the overall K⁺ reabsorptive ability of the kidney. In fact, the nongastric form of the H⁺-K⁺ pump may only be important during pathophysiological conditions, such as K⁺ depletion.

**INVOLVEMENT OF K⁺: ENERGETICS**

In using the energy from ATP hydrolysis, H⁺-K⁺ pumps can establish extremely large pH gradients. For instance, gastric parietal cells (433) can produce gastric secretions containing as much as 140 mM HCl (i.e., pH ~0.7). In parietal cells of rabbit gastric glands, applying omeprazole (which blocks H⁺-K⁺ pumps) reduces pHi by nearly 0.1 in glands stimulated with histamine and IBMX, though not in resting glands. This result implies that the H⁺-K⁺ pump contributes to the maintenance of pHi in stimulated parietal cells.

Functional studies of nongastric H⁺-K⁺ pumps have been performed in vitro using expression systems such as the *Xenopus* oocyte and HEK-293 cells. In such expression systems, the nongastric pumps exchange extracellular K⁺ for intracellular H⁺. However, the pumps may also possess the ability to transport Na⁺ in place of H⁺ (see review, 270).

**Na⁺-Driven Cl⁻-HCO₃⁻ Exchanger**

**GENERAL FUNCTION AND MOLECULAR IDENTITY**

A secondary active transport system that exchanges external Na⁺ and HCO₃⁻ for internal Cl⁻ and H⁺ (or its equivalent, vide infra) was first identified in invertebrate cells such as squid axons (68, 468) and snail neurons (547). Indeed, the work on the squid axon was the first evidence for the dynamic regulation of pHi (61). This transporter makes an important contribution to pHi regulation in mammalian neurons (486). Not certain is the precise identity of the transported acid-base equivalents, inasmuch as pHi measurements cannot clearly distinguish inward movements of HCO₃⁻, CO₃⁻ or NaCO₃ from one another, or from outward movements of H⁺. All four schemes outlined in Fig. 12B (top left) can account for the data. For simplicity, we shall refer to the transporter as the Na⁺-driven Cl⁻-HCO₃⁻ exchanger.

As mentioned earlier and described in greater detail in Chapter 52, investigators have cloned the cDNA encoding the Na⁺-driven Cl⁻-HCO₃⁻ exchanger (NDCBE or SLC4A8) and have also examined the transport properties of the protein (223). When expressed in *Xenopus* oocytes, human NDCBE exhibits all the functional hallmarks of the endogenously expressed transporter. For instance, stimulation of NDCBE activity in oocytes causes an increase in pHi that (1) requires external Na⁺ and HCO₃⁻, (2) can be inhibited by stilbenes, and (3) correlates with a decrease in intracellular Cl⁻ (223). Using the surface-pH measurement technique described previously for examining CO₃⁻ transport by NBCe1, Grichchenko and Boron (221) have provided evidence that NDCBE can also transport CO₃⁻.

**DEPENDENCE ON pH, AND CALCULATION OF ACID EXTRUSION RATES**

Using the approach outlined in Fig. 11B through 11D, Boron et al. (65) separately determined the pHi dependence of the Na⁺-driven Cl⁻-HCO₃⁻ exchanger of barnacle muscle at pH values of 6.8, 7.4, 8.0, and 8.6 (at a fixed [CO₃⁻] of 0.4%). As shown in Fig. 13, the flux is highest at low pH values, and gradually falls toward zero at a pH of ~7.4, the threshold for activating the exchanger. This work was the first to demonstrate a pH-dependent behavior for an acid-extruder; similar work followed on the Na⁺-H⁺ exchanger (see following sections).

The pHi sensitivity of acid extrusion in Fig. 13 is probably not a Michaelis–Menten type of dependence on intracellular H⁺, which would translate into a sigmoid (rather than a linear) acid-extrusion rate versus pHi relationship. The pHi sensitivity in Fig. 13 is probably not a Michaelis–Menten type of dependence on intracellular H⁺, which would translate into a sigmoid (rather than a linear) acid-extrusion rate versus pHi relationship. The pHi sensitivity may reflect an allosteric site for intracellular H⁺ (vide infra).

It is important to recognize that, for two reasons, an analysis of the type shown in Fig. 13 is far more valuable than a simple plot of the pHi recovery rate (dφH/dt) versus pHi. First, because the analysis includes buffering power, the result is a flux rather than merely a rate of change in pHi. This consideration is important when buffering power changes appreciably over the pHi range in question, or if a transporter is to be compared under conditions when buffering power is
expected to vary. Second, the analysis takes into account processes other than the transporter in question; these other processes may contribute to the pH recovery (or lack thereof). Failing to make this correction can lead to a serious over- or underestimation of the flux due to the transporter of interest.

**DEPENDENCE ON pH**

Although the Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchange rate is stimulated at low values of pH\(_0\), it is apparently inhibited at low values of pH\(_i\). For example, both in barnacle muscle and squid giant axons, decreases in pH\(_0\)—produced by lowering [HCO\(_3\)\(^-\)]\(_e\), at a fixed P\(_{CO_2}\)—cause \(J_b\) to decrease (64, 66). However, at least for the squid axon, this apparent inhibition at low pH\(_0\) may be only illusory: when pH\(_0\) is decreased at a fixed [NaCO\(_3\)\(_e\)], the inhibition disappears (64). Thus, at least in squid axons, the transporter per se may be relatively pH\(_i\) insensitive. Most methods of producing acidosis may inhibit Na\(^+-\)driven HCO\(_3\)\(^-\) transport because they simultaneously lower [NaCO\(_3\)\(_e\)].

**INVOLVEMENT OF Na\(^+\), Cl\(^-\), AND HCO\(_3\)\(^-\)**

The Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchanger has an absolute requirement for external Na\(^+\) (545), external HCO\(_3\)\(^-\) (60) or a related species, and internal Cl\(^-\) (467). Removing any one of these three ions from the appropriate side of the membrane completely blocks acid extrusion. Moreover, Na\(^+\) and Cl\(^-\) are actually transported along with acid equivalents (467, 545). The observed stoichiometry is one Na\(^+\) entering the cell, one Cl\(^-\) exiting, and two acid equivalents neutralized intracellularly. This stoichiometry is consistent with all four models presented in Fig. 12B (top left). The prediction that Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchange should be electroneutral is supported by the observations that transport neither alters (56, 544) nor is altered by \(V_m\) (68).

**ENERGETICS**

The models of Fig. 12B (top left) make equivalent energetic predictions, based upon which physiological gradients for Na\(^+\), Cl\(^-\), and HCO\(_3\)\(^-\) should support Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchange with no external source of energy (456). The major predicted energy source is the steep Na\(^+\) gradient, established by the Na\(^+-\)K\(^+\) pump. This model of a secondary active transporter, driven only by ion gradients, is supported by the observation that transport can be reversed by reversing gradients for Na\(^+\) and/or HCO\(_3\)\(^-\) (65, 469, 546). Curiously, however, the transporter in squid axons requires ATP, not as an energy source, but probably for an essential phosphorylation event or as an essential cofactor (62).

**THE pH\(_0\), THRESHOLD**

In barnacle muscle, the activity of the Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchanger falls toward zero as pH\(_0\) approaches a pH threshold of ~7.4 (see Fig. 13). This reduction in transport does to drive pH\(_i\) to ~8.0. Threshold behavior is thus probably due to an allosteric effect of H\(^+\) on the transporter or an essen not reflect the unavailability of energy, however, which is sufficient tial activator. This hypothesis is supported by the observation that the barnacle-muscle Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchanger, in addition to mediating net acid extrusion, also mediates what appears to be Na\(^+-\)Na\(^+\) (469) and Cl\(^-\)-Cl\(^-\) exchange (69). Like acid extrusion, these ancillary transporter activities are also stimulated by reducing pH\(_i\) below the threshold.

**SENSITIVITY TO PHARMACOLOGICAL AGENTS**

The Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchanger is totally and irrreversibly blocked by the stilbene derivatives SITS and DIDS. The site of permanent action is apparently a free amino group on the transporter that reacts with the isothiocyanate moiety of the stilbene to form an N,N-disubstituted thiourea (Edman reaction). However, even stilbene derivatives lacking an isothiocyanato group can inhibit the transporter, although the inhibition is often less than complete and is reversible. Thus, 4,4’-dinitro-2,2’-stilbene disulfonate (DNDS), at a concentration of 1 mM, reversibly inhibits the squid-axon transporter by approximately 80% (63). By analogy to the erythrocyte Cl\(^-\)-HCO\(_3\)\(^-\) exchanger, the Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchanger probably forms a reversible, ionic complex with each of the aforementioned stilbenes. This interaction presumably involves the negatively charged sulfonate groups on the stilbene and cationic moieties on the transporter. The stilbenes with amino-reactive groups (i.e., SITS and DIDS) subsequently form a covalent bond that eventually blocks transport irreversibly. Interesting, the inhibition of squid-axon Na\(^+-\)driven Cl\(^-\)-HCO\(_3\)\(^-\) exchanger by DNDS is apparently competitive with Na\(^+\) as...
well as with HCO$_3^-$; this result is quantitatively consistent with the hypothesis that DND5 actually competes with the NaCO$_3^-$ ion pair for binding (63).

Two nonstilbene amino-reactive agents also inhibit Na$^+$-driven Cl$^-$–HCO$_3^-$ exchange (56). The inhibition by p-isothiocyanato-benzenesulfonate is irreversible, as is its reaction with free amino groups. The inhibition by pyridoxal phosphate is reversible, as is its reaction with free amines. Na$^+$-driven Cl$^-$–HCO$_3^-$ exchange is also reversibly inhibited by relatively high levels (~0.5 mM) of furosemide (68, 69).

**Na$^+$–H$^+$ Exchangers**

**GENERAL FUNCTION AND MOLECULAR IDENTITY**

Na$^+$–H$^+$ exchangers (Fig. 12B, bottom left) have been identified in the plasma membranes of a wide variety of vetebrate cells (for reviews, see [24, 131, 191, 230, 255, 397, 574]). At least two functional classes of Na$^+$–H$^+$ exchangers have been identified. The first, which is apparently the most widespread version, are the Na$^+$–H$^+$ exchangers of nonpithelial cells (7, 103, 226, 228, 378, 379, 413, 458) as well as the basolateral membranes of many epithelial cells (52, 107). This distribution is characteristic of what have been regarded as “housekeeping” transporters (e.g., Na$^+$–K$^+$ pump and Na$^+$–Ca$^{2+}$ exchanger), which are inhibited by relatively low concentrations of five-amino-substituted amiloride analogs, (e.g., ethylisopropyl amiloride [EIPA]). The second class of exchangers is the one characteristically located at the apical membrane of epithelia such as the renal proximal tu-

bule (16, 266, 294, 318, 393, 478, 485). It is inhibited by only relatively high concentrations of the aforementioned amiloride analogs. These two pharmacologically distinct Na$^+$–H$^+$ exchangers have been identified on the apical and basolateral membranes of LLC-PK1 cells (239). A third pharmacological class of Na$^+$–H$^+$ exchange is found in rat astrocytes (80) and hippocampal neurons (36, 47, 436, 486). These cells have an Na$^+$–driven acid extruder that functions in the nominal absence of HCO$_3^-$ and is not inhibited even by high levels of amiloride and/or amiloride analogs. A cDNA encoding a Na$^+$–H$^+$ exchanger (NHE1) was first cloned by Sardet et al. (476), using an expression strategy outlined in Chapter 54. Nine additional NHE isoforms (NHE2 to NHE10) have been identified, all members of the SLC9 family. The sequence comparisons, tissue distributions, and functions of the various NHE isoforms are examined in Chapter 54. In the remainder of this section, we will examine the physiology of Na$^+$–H$^+$ exchange.

**DEPENDENCE ON pH$_i$**

The first convincing evidence that decreases in pH$_i$ stimulate Na$^+$–H$^+$ exchange came from experiments on membrane vesicles, in which low pH$_i$ stimulates amiloride-sensitive Na$^+$ efflux (25). This result, analogous to the Cl$^-$ influx and Na$^+$-efflux data on the Na$^+$-driven Cl$^-$–HCO$_3^-$ exchanger, extends the allosteric-H$^+$-site hypothesis to the Na$^+$–H$^+$ exchanger.

One can also determine the pH$_i$ dependence of the Na$^+$–H$^+$ exchanger in experiments on intact cells. However, an examination of the literature describing the pH$_i$ dependence of Na$^+$–H$^+$ exchangers reveals that many investigators assume that the Na$^+$–H$^+$ exchanger is the only factor contributing to the recovery of pH$_i$ from an acute acid load. In support of this conclusion, they point out that the recovery occurs in the nominal absence of HCO$_3^-$ and is inhibited by blockers of Na$^+$–H$^+$ exchanger. However, there is a serious flaw in this line of reasoning. It is true that, after a cell containing a Na$^+$–H$^+$ exchanger is acutely acid loaded, there is a more-or-less exponential pH$_i$ recovery (e.g., see bcd in Fig. 11A) that may be prevented by pretreating with amiloride analogs. However, this result only proves that an intact Na$^+$–H$^+$ exchanger is required for the pH$_i$ recovery. Such experiments do not address the issue of how background acid-loading processes influence the rate of the pH$_i$ recovery, and thus cannot—by themselves—provide the pH$_i$ sensitivity of the Na$^+$–H$^+$ exchanger.

An analysis of the pH$_i$ sensitivity of Na$^+$–H$^+$ exchange in intact cells requires that one rule out or at least account for background acid-loading processes that oppose the action of the exchange, as discussed above in connection with Fig. 11. In experiments on isolated hepatocytes in the nominal absence of HCO$_3^-$ (82, 530), applying amiloride has no effect on steady-state pH$_i$. This implies that both Na$^+$–H$^+$ exchange and background acid loading are inactive at this pH$_i$ of ~7. During the recovery from an acid load, amiloride analogs block the pH$_i$ recovery, but do not unmask a background acid-loading process. Thus, in these cells, the product of ($\delta^7/p$) and the gross pH$_i$ recovery rate is the true net Na$^+$–H$^+$ exchange rate, which varies with pH$_i$ in a nearly linear fashion (Fig. 14A).

In many other cells, however, applying an amiloride analog causes a sizeable fall in steady-state pH$_i$ (322, 379, 380), as indicated by $de$ in Fig. 11B, implying that both acid-loading and Na$^+$–H$^+$ exchange were active at the original steady-state pH$_i$. A similar EIPA-induced pH$_i$ decrease occurs in renal mesangial cells. The pH$_i$ dependence of the true Na$^+$–H$^+$ exchange rate thus can be computed only after correcting the gross pH$_i$ recovery rate for EIPA-insensitive acid extrusion and EIPA-unmasked acid loading, as discussed in connection with Figs. 11B, 11C, and 11D. The result for the mesangial cell is the curve shown in Fig. 14B, which indicates that the Na$^+$–H$^+$ exchanger is substantially less pH$_i$ sensitive in the physiological range than under conditions of severe acid loading (74).
absence of CO2/HCO3 experiments were performed on single cells (passage 2–5) in the nominal sizeable acidification, indicating that the exchanger is normally active. The exchanger is active in the steady state. Applying EIPA in the steady state unmasks a

cryptotes in the nominal absence of CO2/HCO3 state. The experiments were performed on single freshly disaggregated he-
v
recovery is completed. Thus, the exchanger is inactive in the normal steady state. The experiments were performed on single isolated hepatocytes. (From ref. 74.)

FIGURE 14 Two patterns of pH dependence of the Na+/H+ exchanger. A: The Na+/H+ exchanger of rat hepatocytes exhibits true pH, threshold behavior. Although applying EIPA completely blocks the recovery of pH from an acid load, EIPA has no effect on pH after the pH recovery is completed. Thus, the exchanger is inactive in the normal steady state. The experiments were performed on single freshly disaggregated hepatocytes in the nominal absence of CO2/HCO3. pH was measured using BCECF and a microscope-based fluorometry system. (Used with permission from Boyarsky G, Rosenthal N, Barrett E, Boron WF. Effect of dia-

b: Benzamil is even more active than amiloride against the Na+/H+ exchanger (426).

Effects of Pharmacological Agents

Amiloride (Fig. 15A) is a K+-sparing diuretic that inhibits its apical ENaC Na+ channels in tight epithelia; K values are in the range 0.1 to 1 μM (18, 38). However, at substantially higher concentrations, amiloride also inhibits certain Na+/H+ exchangers (K: 3 to 1000 μM; 39, 351) and Na+/Ca2+ exchange (K: 300 to 1100 μM) (277, 481). The inhibition of Na+/H+ exchange by amiloride has generally been found to be purely competitive with respect to external Na+ (295, 413, 448, 568), although both mixed-type (267) and noncompetitive inhibition (382) have been reported. Because of the competition with Na+, millimolar levels of amiloride generally are necessary to inhibit Na+/H+ exchange at physiological Na+ concentrations (7, 58, 376, 448). Structure-function relationships, which have been re-
reviewed in some detail (38, 39, 190), indicate that different regions of the amiloride molecule alter the molecule's curve with a pK′a of 7.45 (26). Based on kinetic analyses, external H+, Na+, Li+, and amiloride all compete for a common site on the exchanger (for review, see [24]).

Involvement of Na+

Acid extrusion by the Na+/H+ exchanger in intact cells is blocked by removing extracellular Na+ (7, 58, 376). Furthermore, in the presence of extracellular Na+, acid extrusion is accompanied by a rise in intracellular Na+ activity (7, 58, 376) that is blocked when acid extrusion is blocked by lowering pHi (58). Reported values of the apparent K_M for ex-

FIGURE 15 Amiloride and two analogues. A: Amiloride inhibits the Na+ channel in the luminal membrane of many tight epithelia at micro-
molar levels. It inhibits Na+/H+ exchange at approximately millimolar concentrations, and inhibits Na+/Ca2+ exchange at even higher concen-

trations. B: Benzamil is even more active than amiloride against the Na+ channel, but has almost no effect on the Na+/H+ exchanger. C: Ethylisopropylamiloride can be up to 200 times more potent than amiloride against the Na+/H+ exchanger, but is almost without effect on the Na+ channel.
affinity for the apical Na\(^+\) channel, the Na\(^+\)-Ca\(^{2+}\) exchanger, or the Na\(^+\)-H\(^+\) exchanger. Substitutions at the terminal guanidine nitrogen of amiloride, such as the introduction of the benzyl group that produces benzamil (Fig. 15B), can result in compounds with substantially higher affinity for the Na\(^+\) channel, but extremely low affinity for the Na\(^+\)-H\(^+\) exchanger. Conversely, alkyl substitutions at the five-amino position, such as the introduction of ethyl and isopropyl groups that produce EIPA (Fig. 15C), result in compounds with greatly diminished affinity for the apical Na\(^+\) channel, but substantially increased affinity for the Na\(^+\)-H\(^+\) exchanger. Replacement of the Cl\(^-\) at position 6 with Br\(^-\) or I\(^-\) produces compounds with increased affinity for the Na\(^+\)-H\(^+\) exchanger, but decreased affinity for the Na\(^+\) channel and Na\(^+\)-Ca\(^{2+}\) exchanger. HOE compounds such as HOE694 and HOE642 (Cariporide) are relatively new Na\(^+\)-H\(^+\) exchanger inhibitors that have different affinities for several NHE isoforms, with NHE1 being the most sensitive. Similar to amiloride, the base structure of HOE compounds is guanidinium. However, unlike amiloride and its sister compounds, HOE compounds have an attached benzene ring.

As summarized previously, the inhibition produced even by five-amino–substituted derivatives of amiloride (e.g., EIPA), can range from powerful to modest to nil. Furthermore, in rat osteoclasts (439), rat CA1 neurons (47), and rat astrocytes (81) (M.O.B., Frey, and W.F.B., unpublished data), 50 μM EIPA not only fails to inhibit Na\(^+\)-H\(^+\) exchange, it elicits a marked increase in steady-state pH\(_i\). The mechanism responsible for the EIPA-induced increase in pH\(_i\) is presently unknown.

**Electroneutral and Electrogenic Na\(^+\)/HCO\(_3\)\(^-\) Cotransporters**

As discussed earlier, an electronegic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter with a Na\(^+\):HCO\(_3\)\(^-\) stoichiometry of 1:1 is present on the basolateral membrane of proximal tubule cells of the kidney, where it mediates Na\(^+\) and HCO\(_3\)\(^-\) reabsorption, and therefore functions as an acid loader. A member of the SLC4 family, NBCe1 (SLC4A1) is responsible for HCO\(_3\)\(^-\) transport in the proximal tubule. A second transporter, NBCe2 (SLC4A5), which is expressed at high levels in hepatocytes, can also mediate electroneutral Na/HCO\(_3\) cotransport with an apparent stoichiometry of 1:3 (479). However, both NBCe1 (362, 489) and NBCe2 (569) can also operate with a stoichiometry of 1:2, in which case they move Na\(^+\) and HCO\(_3\)\(^-\) into cells, and therefore function as acid extruders. An electronegic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter with a Na\(^+\): HCO\(_3\)\(^-\) stoichiometry of 1:2 (Fig. 12B, top right) has been documented in both invertebrate and mammalian glial cells (see reviews [148, 150, 361, 457]).

An electroneutral Na\(^+\)/HCO\(_3\)\(^-\) cotransporter with a Na\(^+\): HCO\(_3\)\(^-\) stoichiometry of 1:1 (Fig. 12B, bottom right) has been described in sheep Purkinje fibers (138), guinea pig myocytes (184) and vascular smooth-muscle cells (1). Two SLC4 members, NBCn1 (120, 430) and apparently also NCBE (Musa-Aziz, Parker, and W.F.B., unpublished data) mediate electroneutral Na/HCO\(_3\) cotransport. The molecular biology of electronegenic and electroneutral NBCs is presented in more detail in Chapter 52.

**Monocarboxylate Transporters**

**General Function and Molecular Identity of MCTs**

Na\(^+\)-independent transport of monocarboxylates such as lactate occurs by a process of cotransport with H\(^+\) (or exchange with OH\(^-\)) across the plasma membranes of most cell types (see review [424]). The responsible transporters are members of the SLC16 family, which includes 14 members (241). The two best characterized monocarboxylate transporters are MCT1 (SLC16A1) (204) and MCT2 (SLC16A7) (203). However, only MCT1-MCT4 exhibit H\(^+\) coupled monocarboxylate transport. In fact, most of the other family members have yet to be functionally characterized. The sequences of the encoded proteins are predicted to have 12 transmembrane (TM) segments, with a large intracellular loop between TM6 and TM7 and greatest conservation in the predicted transmembrane regions. Although both MCT1 and MCT2 have broad tissue distributions, in the kidney, MCT1 is expressed on the basolateral membrane of proximal tubule cells, and MCT2 is expressed on the basolateral membrane of medullary collecting duct cells (203).

**Substrate Specificity and Energetics of the MCTs**

MCT isoforms appear to have different affinities for the same substrates. For example, both MCT1 and MCT2 transport lactate and pyruvate (203), but MCT2 has a much higher apparent affinity for pyruvate (335).

In all cells examined, H\(^+\)/X\(^-\) cotransport appears to be electroneutral (155, 424, 504). Because MCT-mediated transport of monocarboxylates involves cotransport with H\(^+\), the net driving force depends on the gradients of both monocarboxylate and H\(^+\) across the plasma membrane. Depending on the direction of the net driving force, MCTs can mediate either the influx or efflux of monocarboxylates. As described in the following sections, MCT1 on the basolateral membrane of renal proximal tubule cells acts in conjunction with an apical Na\(^+\)/ monocarboxylate (Na\(^+\)/X\(^-\)) cotransporter to facilitate transepithelial lactate reabsorption.

**Effects of Pharmacological Agents on MCTs**

MCT1 and MCT2, but not MCT3 and MCT4, are inhibited substantially by α-cyano-4-hydroxycinnamate (CHC) (203, 241, 606). However, because CHC has yet to be widely tested on other classes of transporters, it is not clear whether this compound is specific for MCTs.
THE Na⁺/MONOCARBOXYLATE COTRANSPORTER

The Na⁺/X⁻ cotransporter is rather nonspecific, capable of transporting D- and L-lactate, pyruvate, acetate, and other monocarboxylates. The cotransporter is found in apical membranes of both the proximal tubule and small intestine (504, 595). The Na⁺/X⁻ cotransporter appears to be electrogenic (moving more Na⁺ than X⁻) in mammals (32, 364, 473, 563), but electroneutral in amphibians (504). Investigators have found that one member of the SLC5 family of “Na/glucose cotransporters,” namely SLC5A8, encodes an electronegative sodium-coupled monocarboxylate transporter (SMCT) (122, 220, 373, 422). In addition, zebrafish SLC5A12 encodes an electroneutral SMCT (422). As expected, the transporters are relatively nonselective for monocarboxylates, although substrate affinities can vary among the transporters.

INTERACTION OF Na⁺/X⁻ AND H⁺/X⁻ COTRANSPORTERS

Some proximal-tubule cells possess both a Na⁺/X⁻ cotransporter at the apical membrane and an MCT at the basolateral membrane (Fig. 12C). Acting in concert, these two monocarboxylate transporters can extrude substantial amounts of acid while reabsorbing monocarboxylates (389, 504).

The first step of the acid-extruding process is the coupled entry of Na⁺ and X⁻ across the apical membrane, an example of secondary active transport. Because the pKᵦ govern- ing the dissociation of monocarboxylic acids (HX ⇌ H⁺ + X⁻) is generally far below pHᵦ, very little of the entering X⁻ can combine with H⁺ to form HX, and thus Na⁺/X⁻ cotransport by itself has very little effect on pHᵦ. In the second step, X⁻ entering via luminal Na⁺/X⁻ cotransport rapidly exits across the basolateral membrane via MCT—along with H⁺. In this system, basolateral H⁺/X⁻ cotransport is an example of tertiary active transport because the extrusion of H⁺ is driven by an X⁻ gradient that is, in turn, established by a secondary active transporter.

The above system of monocarboxylate transport is expected to have effects on transepithelial transport and pHᵦ regulation:

1. Na⁺ reabsorption. Following the coupled uptake of Na⁺ and X⁻ across the apical membranes, the extrusion of Na⁺ across the basolateral membrane by the Na⁺-K⁺ pump contributes to transepithelial Na⁺ reabsorption. Indeed, acetate promotes volume reabsorption (480).

2. X⁻ reabsorption. Following the coupled uptake of Na⁺ and X⁻ across the apical membrane, H⁺ and X⁻ exit across the basolateral membrane. The net effect is the transepithelial reabsorption of monocarboxylate from lumen to blood.

3. Luminal H⁺ secretion. When acetate is added to the lumen of the rabbit S3 proximal tubule, pHᵦ rises markedly, probably due to the apical uptake of X⁻ via the Na⁺/X⁻ cotransporter, followed by the apical efflux of HX, presumably by nonionic diffusion or an MCT-like transporter. The net effect is apical H⁺ secretion and luminal acidification (208).

4. Extrusion of H⁺ across basolateral membrane. H⁺ and X⁻ exit across the basolateral membrane represents a net extrusion of acid that causes a substantial rise in pHᵦ (389, 504). As far as pHᵦ regulation is concerned, the monocarboxylate transporters masquerade as an amiloride-insensitive/monocarboxylate-dependent “Na⁺-H⁺ exchanger.”

THE REGULATION OF pHᵦ

In the preceding sections, we have discussed a wide range of factors that influence pHᵦ. In this and the following section, we will examine how these factors interact to produce transient changes in pHᵦ, and, finally, to establish a steady-state pHᵦ.

Fundamental Law of pHᵦ Regulation

What determines the stability of pHᵦ? It is intuitively obvious that pHᵦ will remain constant as long as the rate of acid extrusion (including uptake of alkali) out of the cytoplasm (Jₑ) is equal to the rate of acid loading (including efflux of alkali) into the cytoplasm (Jᵢ). Thus, pHᵦ will rise if Jₑ exceeds Jᵢ, and pHᵦ will fall if Jᵢ exceeds Jₑ. The rate of pHᵦ change is proportional to the difference between Jₑ and Jᵢ, proportional to the surface-to-volume ratio (p), but inversely proportional to buffering power:

$$\frac{dpH}{dt} = \frac{p}{\beta} (Jₑ - Jᵢ)$$

(Eq. 39)

Equation 39 makes good intuitive sense. If Jₑ = Jᵢ, then dpH/dt = 0, and the cell is in a steady state with respect to pHᵦ. Should Jₑ exceed Jᵢ, pHᵦ must increase because dpH/dt will be greater than zero. Furthermore, the rate of this alka- linization is expected to increase as the difference between Jₑ and Jᵢ increases. If Jₑ and Jᵢ are unequal, then the rate of pHᵦ change must be proportional to the surface-to-volume ratio (p). Thus, pHᵦ transients tend to be faster in smaller cells. Finally, the rate of pHᵦ change must be strongly influenced by β. If β were infinite, then pHᵦ would be fixed, regardless of Jₑ and Jᵢ. Conversely, if β approached zero, then even a small difference between Jₑ and Jᵢ would cause pHᵦ to change very rapidly.

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1 We define acid-extrusion and acid-loading rates as fluxes (J), with the units of moles per unit area of cell surface, per unit time. In practice, such a definition is practical for calculating values from experiments only if the cell has a very simple geometry (e.g., cylinder in the case of an axon). For most mammalian cells, which have complicated geometries, investigators express acid-loading and acid-extrusion rates in what we would term "pseudofluxes" (φ) (45), with the units of moles per unit volume of cytoplasm, per unit time. In the case of a pseudosurface, the surface-to-volume ratio is folded into the φ value. One must use caution in dealing with φ values, which can change with cell swelling or shrinkage, even without a change in the true underlying acid-base "flux."
Factors That Influence the Steady-State pH

A Graphical Representation of Eq. 39

Figure 16 is a plot of $J_E$ and $J_L$ as a function of $pHi$ for a hypothetical cell. The shape of the $J_E$ curve is modeled after that reported for the Na$^+$/H$^+$ exchanger of cultured mesangial cells (74), whereas the shape of the $J_L$ curve is modeled after that reported for background acid loading in renal mesangial cells (74), or Cl$^-$–HCO$^-$ exchange in several cells (76, 404). It is clear from Eq. 39 that $pHi$ can be in a steady state (i.e., $dpHi/dt = 0$) only if $J_E$ and $J_L$ are equal. Thus, in Fig. 16, the steady-state pH is determined by the intersection of the $J_E$ and $J_L$ curves. By using the graphical model in Fig. 16, one can evaluate how steady-state pH is altered by either acid-base disturbances, which we will discuss below, or by other factors such as hormones, mitogens, and oncogenes. Some examples will be presented in the following paragraphs, from which the following three key concepts will emerge.

Buffering Power Does Not Influence Steady-State pH

Buffering is important for cells because it reduces the magnitude of the pH excursions that result from acute (i.e., one-time-only) acid and alkali loads. However, buffering does not prevent pH changes; it only minimizes them. Moreover, after an acute acid or alkali load, buffering cannot return pH to normal. As we will see in the next paragraph, it is the acid-extruding mechanisms that are responsible for returning pH toward normal. According to Eq. 39, $\beta$ only influences the rate of pH recovery from such an acid load.

Acute Acid or Alkali Loads Do Not Affect Steady-State pH

Consider the consequences of an acute acid load (e.g., an injection of H$^+$ or an influx of a weak acid that leads to the intracellular generation of H$^+$). The vast majority of H$^+$ introduced into the cell is neutralized by buffers (the small amount of unbuffered H$^+$ is responsible for the pH decrease). pH can return to normal only when the entire acid load—both the additional free H$^+$ and the added H$^+$ that is reversibly neutralized by buffers—is extruded from the cell. During such a pH recovery, acid extruders remove free H$^+$ from the cytoplasm, and buffers then partially replenish these extruded protons. Thus, during the pH recovery, the added protons move from the buffers to the cytoplasm, from where the acid-extruders translocate them to the outside of the cell. If there has been no fundamental change in the properties of the acid loaders and acid extruders, then pH will return precisely to its initial value. Thus, by themselves, acute acid and alkali loads produce only transient changes in pH.

Only Fundamental Changes in Acid-Extruding and Acid-Loading Processes Can Affect Steady-State pH

Consider the consequences of modifying the pH dependence of an acid-extruding process in such a way that the intersection of the $J_E$ and $J_L$ curves in Fig. 16 is altered. Such a change in transporter kinetics would produce a shift in steady-state pH. Note that, whereas a change in the $J_E$ and/or $J_L$ curves is required for a shift in steady-state pH, the $J_E$ and $J_L$ curves can shift without a change in steady-state pH. For example, if there were appropriate offsetting changes in both curves, then it would be possible for the steady-state pH to remain unaltered, even though $J_E$ and $J_L$ were modified.

Examples of Intracellular Acid-Base Disturbances

Acute Intracellular Acid Load

Figure 17A illustrates the time course of pH in a hypothetical experiment in which we acutely acid load a cell (segment $abc$) and monitor the subsequent pH recovery ($bcd$). Figure 17B is reproduced Fig. 16 and shows the pH dependencies of $J_E$ and $J_L$, as well as $J_E$ and $J_L$ values at different times during segment $abcd$ in Fig. 17A. As described previously, the intersection of the $J_E$ and $J_L$ curves at point $a$ in Fig. 17B determines the initial steady-state pH, where $dpHi/dt = 0$.

We could regard the acute acid load as a square-wave increase in $J_L$. The product of the width (i.e., duration) of the square wave, the height of the square wave, and the surface-to-volume ratio is the amount of acid we are loading into the cell. Dividing this amount by $\beta$ yields the magnitude of the pH decrease, which is represented in Fig. 17A as an instantaneous fall in pH, $ab$. This imposed decrease in pH causes $J_E$ to rise ($b$ on the $J_E$ curve) and $J_L$ to fall ($b$ on the $J_L$ curve). According to Eq. 39, because $J_E$ now exceeds $J_L$, pH must increase. However, as the pH recovery proceeds ($bcd$), $J_E$ gradually falls and $J_L$ rises. Eventually, $J_E$ and $J_L$ come into balance at point $d$, which is identical to point $a$. Thus, as long as an acute intracellular acid load does not produce fundamental changes in the kinetics of acid extrusion or acid loading, the effect on pH is only transitory. Stated differently, if the pH dependencies of $J_E$ and $J_L$ in Fig. 17B are unchanged, then steady-state pH also is un-
Initially, pHᵢ is described by point a in panel no fundamental change in the kinetics of acid extrusion and acid loading. They reach their original values at point d.

During this recovery, Jₑ gradually decreases and Jᵢ gradually increases until they once again come into balance at point d, where pHᵢ now stabilizes. If β does not depend steeply on pHᵢ, then the rate of pHᵢ decline during bcd will be greatest at point b, where the difference (Jₑ - Jᵢ) is greatest. Thus, a chronic inhibition of acid extrusion causes steady-state pHᵢ to fall.

**CHRONIC INHIBITION OF ACID LOADING**

Imagine that our hypothetical cell, initially in a steady state described by point a in Fig. 17E and Fig. 17F, is treated with sufficient DIDS (an inhibitor of Cl⁻-HCO₃⁻ exchange) to reduce Jₑ, to approximately one eighth of its original level at all pHᵢ values in Fig. 17D. Thus, immediately after this reduction in Na⁺-H⁺ exchange rate, Jₑ is unchanged (point b on the Jₑ curve), but Jᵢ is greatly reduced (point b on the Jᵢ curve). According to Eq. 39, because Jᵢ now exceeds Jₑ, pHᵢ must slowly fall (bcd in Fig. 17C). However, as pHᵢ declines, Jₑ rises and Jᵢ falls (e.g., point c in Fig. 17D). Eventually, Jₑ and Jᵢ come into balance at point d, where pHᵢ now stabilizes.

**FACTORS INFLUENCING pHᵢ**

**Interaction of pHᵢ and pHₓ**

It is well established that changes in pHᵢ can affect pHₓ, and also that changes in pHₓ can be accompanied by changes in pHᵢ (444, 549). Such interactions between intra- and extracellular acid-base metabolism are almost certainly mediated by changes in acid-base transport across the cell membrane. We have already discussed several such transport processes, including (1) nonionic diffusion of weak acids and bases (including metabolites), (2) passive flux of charged weak acids and bases through channels, and (3) acid-base movement via a variety of transporters. In the remainder of this section, we will examine how an alteration on one side of the cell membrane can alter transport and thus produce a pH change on the other side.

**EFFECTS OF THE "CLASSIC" CHANGES IN EXTRACELLULAR ACID-BASE STATUS ON pHᵢ**

One can define at least six simple disturbances of extracellular acid-base status, each of which has a unique effect on pHᵢ. Four of these are the "classic" extracellular disturbances: metabolic acidosis and alkalosis and respiratory acidosis and
alkalosis. The other two extracellular acid-base disturbances, isohydric hyper- and hypocapnia, are unusual in that they produce alterations in pH\textsubscript{i} with no change in pH\textsubscript{o}.

**Metabolic Acidosis** A sudden extracellular acidification produced by a reduction of [HCO\textsubscript{3}\textsuperscript{−}], at a fixed P\textsubscript{CO\textsubscript{2}}, is expected to alter pH\textsubscript{i} only by mechanisms that we shall describe as “chronic.” The reductions in pH\textsubscript{i}, and/or [HCO\textsubscript{3}\textsuperscript{−}], are expected to inhibit acid-extrusion processes, but stimulate acid-loading processes. Such kinetic changes could be produced by alterations in any rate-determining parameter, such as pH\textsubscript{eq} [Na\textsuperscript{+}][Na\textsuperscript{+}], binding constants, or the density of transporters. The net effect is expected to be a gradual but sustained decline in pH\textsubscript{i}, similar to that schematized in Fig. 17C. The modeling of this problem is similar to that described in Fig. 17D, except that not only would \(J_{E}\) decrease, but \(J_{L}\) would increase as well. The time course of the pH\textsubscript{i} decline depends on \(\beta\) and on the pH\textsubscript{i} dependencies of \(J_{E}\) and \(J_{L}\). The actual value of the new steady-state pH\textsubscript{i} would, of course, be independent of \(\beta\).

The predictions of the foregoing analysis have been confirmed experimentally. Measurements of steady-state pH\textsubscript{i} with DMO indicate that metabolic acidosis lowers pH\textsubscript{i} in mammalian skeletal and cardiac muscle (3, 456). The time course of this intracellular acidification has also been monitored with pH-sensitive microelectrodes in several vertebrate cells (8, 59, 149, 169).

**Metabolic Alkalosis** A sudden extracellular alkalization produced by raising [HCO\textsubscript{3}\textsuperscript{−}], at a fixed P\textsubscript{CO\textsubscript{2}}, is expected to have chronic effects on acid extrusion, acid loading, and pH\textsubscript{i} that are opposite to those described above for metabolic acidosis. Metabolic alkalosis generally produces a gradual but sustained pH\textsubscript{i} increase, similar to that schematized in Fig. 17E. The modeling of this problem would be similar to that of Fig. 17E, except that not only would \(J_{E}\) increase, but \(J_{L}\) would decrease as well. Steady-state pH\textsubscript{i} measurements with DMO in mammalian muscle (3, 251) have confirmed the foregoing prediction that pH\textsubscript{i} should rise during metabolic alkalosis.

**Respiratory Acidosis** A sudden extracellular acidification produced by an increase in P\textsubscript{CO\textsubscript{2}}, is expected to alter pH\textsubscript{i} by both acute and chronic mechanisms. In the laboratory, it is possible to produce a respiratory acidosis in which [HCO\textsubscript{3}\textsuperscript{−}], is constant. However, in vivo, respiratory acidosis is always accompanied by a rise in [HCO\textsubscript{3}\textsuperscript{−}]. Regardless of the effect on [HCO\textsubscript{3}\textsuperscript{−}], the “acute” effect of respiratory acidosis is produced by the influx of CO\textsubscript{2}, which elicits a rapid fall in pH\textsubscript{i}. This represents an acute intracellular acid load from which the cell would fully recover if the kinetics of acid-extruding and acid-loading mechanisms were not fundamentally altered by the accompanying fall in pH\textsubscript{eq}. The expected “chronic” effects of respiratory acidosis are qualitatively the same as for metabolic acidosis: an inhibition of \(J_{E}\) and a stimulation of \(J_{L}\). These chronic effects will tend to lower steady-state pH\textsubscript{i}, as in metabolic acidosis. Indeed, pH\textsubscript{i} determinations by the DMO technique (3, 456) show that respiratory acidosis does reduce steady-state pH\textsubscript{i}. In principle, the time course of the pH\textsubscript{i} decrease could be predicted from our discussion of CO\textsubscript{2}-induced acidifications (the acute effect) and from Eq. 39 (the chronic effect). Thus, initial pH\textsubscript{i}, initial and final P\textsubscript{CO\textsubscript{2}}, and the kinetics of acid extrusion and acid loading would all influence the time course of acidification. All these parameters except \(\beta\) would also influence the final steady-state pH\textsubscript{i}. After the acute effect (i.e., the rapid CO\textsubscript{2}-induced pH\textsubscript{i} fall), respiratory acidosi could then produce (1) a slower but sustained pH\textsubscript{i} decline (chronic effect), (2) no further pH\textsubscript{i} change (i.e., immediate stabilization), or (3) a pH\textsubscript{i} recovery that is partial, complete or—at least in principle—even exaggerated. Which of the three steady-state outcomes we observe depends upon the relationship between \(J_{E}\) and \(J_{L}\).

**Respiratory Alkalosis** The pH\textsubscript{i} changes produced by respiratory alkalosis, and the mechanisms effecting these changes, are opposite those produced by respiratory acidosis. The effect on acid extrusion is noteworthy because the imposed rise in pH\textsubscript{eq} is expected to stimulate acid extrusion, whereas the resultant increase in pH\textsubscript{i} (caused by the efflux of CO\textsubscript{2}) is expected to inhibit or even totally block acid extrusion. Thus, the expected time course of pH\textsubscript{i} after its rapid initial rise could be (1) a further, slower increase to a new steady state; (2) no change; or (3) a decline. Studies with pH-sensitive microelectrodes have demonstrated the first pattern in mouse skeletal muscle (8), and steady-state pH\textsubscript{i} measurements have confirmed that respiratory alkalosis causes a sustained elevation of pH\textsubscript{i} (3, 456).

**Isohydric Hypercapnia** Raising P\textsubscript{CO\textsubscript{2}} at constant pH\textsubscript{i} (i.e., raising P\textsubscript{CO\textsubscript{2}} and [HCO\textsubscript{3}\textsuperscript{−}] proportionally) produces a rapid fall in pH\textsubscript{i} (61, 543), due to the influx of CO\textsubscript{2}, just as does respiratory alkalosis (vide supra). However, for at least two reasons, the long-term influences on pH\textsubscript{i} can be very different from those produced by respiratory acidosis. First, because pH\textsubscript{i} is fixed, one expects no immediate inhibition of acid extrusion or stimulation of acid loading. Thus, the cell should be better able to resist the acute acid load caused by the influx of CO\textsubscript{2}. Second, because [HCO\textsubscript{3}\textsuperscript{−}] is higher in isohydric hypercapnia than respiratory acidosis, acid loading due to the passive and carrier-mediated efflux of HCO\textsubscript{3}\textsuperscript{−} should be lower than during respiratory acidosis. Third, the aforementioned increase in [HCO\textsubscript{3}\textsuperscript{−}], could also stimulate HCO\textsubscript{3}\textsuperscript{−}-dependent acid-extrusion (e.g., Na\textsuperscript{+}-driven Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} exchange). According to recent data, raising [CO\textsubscript{2}] per se (i.e., in the nominal absence of HCO\textsubscript{3}\textsuperscript{−}) can stimulate H\textsuperscript{+} extrusion from proximal-tubule cells (618). Thus, although isohydric hypercapnia is expected to elicit the same initial, CO\textsubscript{2}-induced fall in pH\textsubscript{i} as respiratory acidosis, the final steady-state pH\textsubscript{i} should be higher, as has been confirmed on mammalian skeletal muscle (3).

The time course of pH\textsubscript{i} during isohydric hypercapnia depends on the four previously mentioned factors (i.e., \(\beta\), initial pH\textsubscript{i}, initial and final P\textsubscript{CO\textsubscript{2}}, and the kinetics of acid extrusion and acid loading). In salamander proximal-tubule cells, pH\textsubscript{i} does not recover from the initial fall, due to the very high HCO\textsubscript{3}\textsuperscript{−} efflux across the basolateral membrane, via NBC (59). In cells with lesser degrees of HCO\textsubscript{3}\textsuperscript{−}-dependent
acid loading, pH_i makes a partial or even complete recovery (57, 456, 591). Finally, in cells with dominant HCO_3^- dependent acid-extrusion mechanisms, such as smooth muscle (6) and renal mesangial cells (75), isohydric hypercapnia increases steady-state pH_i. In both dissociated CA1 neurons (486, 517) and cultured astrocytes (49) from the rat hippocampus, the degree of this pH_i increase becomes less and less at progressively higher initial values of pHe. The pH_i increase produced by isohydric hypercapnia in the rabbit proximal tubule (390) may reflect stimulation of apical Na^+-H^+ exchange and H^+ pumping (113, 114), likely involving sensors for basolateral CO_2 and HCO_3^- (618).

**Isohydric Hypocapnia** Lowering P_CO_2 at constant pH_i, (i.e., lowering P_CO_2 and [HCO_3^-], proportionally) produces an initial rise in pH_i due to CO_2 efflux, as in respiratory alkalosis. The long-term influences on pH_i are the opposite of those described previously for isohydric hypercapnia. Thus, the final steady-state pH_i is expected to be somewhat lower in isohydric hypocapnia than in respiratory alkalosis, as confirmed for mammalian skeletal muscle (4). The time course of changes in pH_i during isohydric hypocapnia has been monitored in several cells, especially during the transition between normal P_CO_2/HCO_3^- to nominally CO_2/HCO_3^- free conditions. As expected, the reduction in P_CO_2 at constant pH_i causes an abrupt increase in pH_i (due to CO_2 efflux), followed by a partial return of pH_i toward normal (probably due to inhibition of acid extrusion and stimulation of acid loading at high pH_i). In mesangial cells, the switch from 10% CO_2/50 mM HCO_3^- (pH_i = 7.4) to 5% CO_2/25 mM HCO_3^- (pH_i = 7.4) causes an abrupt increase in pH_i that represents an acute intracellular alkali load. The subsequent recovery of pH_i, due primarily to Cl^-/HCO_3^- exchange, can then be examined at physiological levels of extracellular CO_2 and HCO_3^- (200). A similar approach has been used by Ou-yang et al. (407) to examine Cl^-/HCO_3^- exchanger activity in neurons cultured from the rat cortex.

**USE OF OUT-OF-EQUILIBRIUM CO_2/HCO_3^- TO MAKE ISOLATED CHANGES IN EXTRACELLULAR [CO_2], [HCO_3^-], AND pH ONE AT A TIME**

In 1995, Zhao et al. (614) introduced a rapid-mixing technique that allows one to create out-of-equilibrium (OOE) CO_2/HCO_3^- solutions with virtually any combination of [CO_2], [HCO_3^-], and pH_i in the pathophysiological pH range. The approach is to mix the outputs of two syringes—one containing a different combination of equilibrated [CO_2], [HCO_3^-], and pH_i—and then rapidly (<200 ms) deliver the newly mixed solution to the cells, while continuously sweeping away the solution after it has contacted the cells.

**SENSORS FOR EXTRACELLULAR [CO_2], [HCO_3^-], AND pH_i**

Zhou et al. (618) used the powerful OOE approach (see previous section) to alter, systemically, the basolateral (BL) [CO_2], [HCO_3^-], and pH—one at time—in experiments on isolated, perfused rabbit proximal tubules. They found that increasing [CO_2]_BL—while holding [HCO_3^-]_BL and pH_bl fixed—caused an increase in the rate of HCO_3^- reabsorption (measured during a period of ~20 minutes). This is the appropriate response to the “respiratory” part of acute respiratory acidosis. Conversely, increasing [HCO_3^-]_BL—while holding [CO_2]_BL and pH_bl fixed—caused the rate of HCO_3^- reabsorption to decrease. This is the appropriate response to the “metabolic” part of acute metabolic acidosis. Finally, they found that increasing pH_bl—while holding [CO_2]_BL and [HCO_3^-]_BL fixed—had no effect on the rate of HCO_3^- reabsorption. In other words, at least during acute stresses, the proximal tubule is incapable of responding to changes in pH_bl per se. Rather, the tubule responds to changes in the concentrations of the two major buffers, CO_2 and HCO_3^-.

These data are consistent with the hypothesis that the proximal tubule has some sort of sensor or sensors that detect extracellular levels of CO_2 and extracellular HCO_3^-.

Thus, the pH of the bulk extracellular fluid (pH_ECF) is closely regulated by the respiratory and renal systems. Nevertheless, the movement of acids and bases across cell membranes can modify pH_ECF. For example, in the central nervous system, where the neurons and glial cells are in close apposition to one another, acid-base transport across the cell membranes can profoundly alter pH_i (see reviews, 117–119, 438). Acid-base movement across cell membranes can result in parallel or reciprocal changes in pH_i.
and pH.<sub>i</sub>. Parallel decreases in pH and pHe can occur when an increased rate of lactic acid production (which lowers pH) leads to the subsequent efflux of lactic acid via H<sup>+</sup>/Lac<sup>-</sup> cotransport (which lowers pHe). The efflux of lactic acid minimizes the fall in pH. Reciprocal increases in pH and decreases in pHe occur in gallbladder epithelial cells due to Na<sup>+</sup>-H<sup>+</sup> exchange (444), and in depolarized snail neurons due to a voltage-activated conductance pathway (549). In both of these examples, stabilization of pH occurs at the expense of pHe homeostasis.

### Role of the Extracellular Fluid in pH Regulation

It is well established that changes in pHe will elicit reciprocal changes in pH. A general rule of thumb: the magnitude of a pHe change will be approximately one third the magnitude of the pH change. However, ΔpH/ΔpHe can vary considerably among cells, being as high as 0.7 in mesenteric vascular smooth muscle (29), carotid-body glomus cell (86), and CNS neurons (71). As noted previously, extracellular metabolic acidosis causes a sustained fall in pH.<sub>i</sub>. The overall effect of this acidosis on acid–base transport is therefore a net transfer of acid from the ECF to the ICF of all accessible cells, causing pH<sub>i</sub> to increase somewhat toward normal, but at the expense of causing pH to decrease throughout the organism. One interpretation of these reciprocal changes in pH and pHe is that cells act as a pH buffer for the ECF, preventing large fluctuations in pH.<sub>e</sub>. For example, “healthy” cells taking up H<sup>+</sup> minimize the fall in pH.<sub>e</sub> due to the release of lactic acid by a subpopulation of hypoxic cells. The partial uptake of this acid from the ECF by the healthy cells may thus be viewed as a mechanism for distributing the acid insult among all cells accessible to the ECF until such time that the renal and respiratory systems can correct the acid–base disturbance.

In the steady state in most animals, there is a net transfer of metabolically generated acid from cells to the ECF, and from the ECF to the urine, as depicted in Fig. 18. In the kidney, tubules from both the proximal nephron (16, 58, 59, 318) and distal nephron (493, 585) are composed of cells that regulate pH<sub>e</sub> much as would nonepithelial cells: They respond to intracellular acid loads by extruding acid. However, these cells are unique in that HCO<sub>3</sub><sup>-</sup> efflux, which provides the major intracellular acid load, is limited to the cells’ basolateral (or blood side) membrane (9, 15, 17, 59, 319, 607). On the other hand, acid extruders (e.g., the Na<sup>+</sup>-H<sup>+</sup> exchanger and H<sup>+</sup> pump) are generally most active at the luminal membrane (16, 268, 393). Thus, the acid extruded into the ECF from nonrenal cells enters renal tubule cells via basolateral HCO<sub>3</sub><sup>-</sup> efflux, lowering renal tubule pH<sub>i</sub>. Presumably stimulated by this pH<sub>i</sub> decrease, luminal acid extruders move the acid into the tubule lumen. Thus, the regulation of pH<sub>i</sub> by certain renal tubule cells ultimately leads to acid excretion by the kidney. The renal tubule cells therefore provide the final link between pH<sub>i</sub> and pHe: pH<sub>i</sub> regulation by renal tubule cells is responsible for pHe regulation, which in turn makes possible pH<sub>e</sub> regulation by the body’s other cells.

### Temperature Changes

Decreasing the temperature generally produces a pH increase in the blood and intracellular fluid (440, 456). In principle, a temperature change could alter pH, in at least three ways: (1) by modifying the pK<sub>a</sub> of pH buffers, (2) by modifying the pK<sub>a</sub> of enzymes or transporters involved in acid–base homeostasis, or (3) by changing the activation energy for an enzymatic reaction or transport process.

### Effect of Temperature Changes on the pK<sub>a</sub> of Buffers

The integrated form of the van’t Hoff equation predicts that, in a closed system (i.e., for a fixed total amount of buffer), a buffer’s pK<sub>a</sub> should vary inversely with temperature:

\[
\text{pK}_1 - \text{pK}_2 = \frac{\Delta H}{R \ln 10} \left[ \frac{1}{T_1} - \frac{1}{T_2} \right] \tag{40}
\]

where ΔH is the heat of ionization, R is the universal gas constant, T<sub>1</sub> and T<sub>2</sub> are two absolute temperatures, and the subscripts 1 and 2 refer to pK<sub>a</sub> values at the corresponding two temperatures. If a solution contains only one buffer, then a temperature change will produce parallel shifts in pK<sub>a</sub> and pH. In the case of a solution containing several buffers (e.g., intracellular fluid), the temperature-induced pH shift depends on the sum of the individual pK shifts, each weighted by the buffer’s relative contribution to total buffering (87).

Reeves and Malan (441) have analyzed the pH<sub>e</sub> changes produced by temperature shifts in frog muscle in vivo. Over the same temperature range, the in vivo pH<sub>e</sub> values in these
EFFECT OF TEMPERATURE CHANGES ON ENZYMES AND TRANSPORTERS

The effects of temperature changes on acid-base transporters and other enzymes affecting pHi are not well documented. The effects are most likely of two sorts: (1) A temperature-induced shift in the pKₐ of an ionizable group on an enzyme or transport protein could affect the kinetics of the reaction or transport process, or (2) raising the temperature speeds reactions by increasing the energy of the reactants. As far as pKₐ shifts are concerned, a change in temperature would be expected to alter the ionization state of titratable groups on proteins, with effects being greatest for groups with pKₐ values similar to the prevailing pH. For instance, a rise in temperature would lower the pKₐ of an amino group of a protein, thereby reducing the fraction of molecules with a positive charge at that position. Conversely, the fraction of molecules with a negatively charged carboxyl group would rise. Such alterations in ionization could lead to conformational changes that could have major effects on protein function.

As far as direct effects of temperature changes on reaction rates are concerned, enzymatic reaction rates as well as passive and active fluxes of ions all are expected to fall with decreasing temperature, although to varying degrees. In mouse soleus muscle, reducing the temperature from 37°C to 28°C causes the acid-extrusion rate to fall by 65% (7). Similarly, in cultured astrocytes from rat hippocampus, reducing the temperature from 37°C to room temperature causes the acid-extrusion rate elicited by exposing the cells to CO₂/HCO₃⁻ to fall by ~40% (M.O.B. and W.F.B., unpublished data). In a study on cultured fetal rat hippocampal neurons, Baxter and Church (36) have presented evidence that the Na⁺-H⁺ exchanger contributes to the maintenance of steady-state pH i at 18–22°C, but not at 37°C. In these neurons, increasing temperature may activate the Na⁺-H⁺ exchanger more than the Na⁺-driven Cl⁻-HCO₃⁻ exchanger. In addition to cellular changes brought about by temperature shifts, whole-body physiological changes (e.g., hormone levels, P̂CO₂, plasma [HCO₃⁻]) may also influence pH i.

METABOLIC INHIBITORS AND ANOXIA/HYPOXIA

Metabolic inhibitors, anoxia/hypoxia, and ischemia generally produce slow decreases in pH i. Metabolic inhibitors have been examined most closely in isolated nerve and muscle preparations using pH-sensitive microelectrodes. Both azide and 2,4-dinitrophenol (DNP) produce two-stage decreases in pH i (61, 543): an abrupt fall followed by a slower one. The abrupt pH i decrease is probably due to the influx and dissociation of the protonated weak-acid form of the inhibitor, whereas the slower pH i decrease may be caused by a net increase in the production of acidic metabolites, confounded perhaps by a decrease in the rate of acid extrusion. Squid axons that are exposed to hydrocyanic acid (HCN ⇌H⁺ + CN⁻; pKₐ = ~10) display only a slow decrease in pH i (61), the origin of which is likely the same as those of the slow phase of the azide and DNP-induced acidifications. The failure of HCN to produce a rapid pH i decrease in squid axons is due to its high pKₐ at physiological pH i, only a small fraction of incoming HCN dissociates into CN⁻ and H⁺.

Ischemia and anoxia/hypoxia can also elicit decreases in pH i due to the lactic acid production that results from increased glycolysis in the absence of oxidative phosphorylation (252, 604). For example, using ³¹P-NMR to measure the pH i of beating rat hearts, Gadian et al. (196) observed that ischemia produced a gradual fall in pH i from 7.05 to 6.2 in 13 minutes. However, in hearts previously depleted of glycogen, the pH i fall triggered by a subsequent episode of ischemia is reduced by ~50% (206). Similarly, preincubating the hearts in 2-deoxyglucose, which inhibits phosphorylase b and thus glycogen breakdown, reduces the ischemia-induced pH i decrease by ~25%. Although anoxia/hypoxia might be expected to decrease pH i by interfering with active-transport processes that extrude acid, the effects of anoxia/hypoxia appear to be more complex and are likely to depend on several factors including the cell type, the experimental preparation (e.g., cells in situ vs cells in culture), duration of anoxia/hypoxia, and the presence versus absence of CO₂/HCO₃⁻ as well as other parameters that influence the relative activity of various acid-base transporters. For example, in the isolated turtle heart, anoxia inhibits a DIDS-sensitive, Na⁺- and HCO₃⁻-dependent mechanism by ~50% (499). In contrast, acute anoxia appears to stimulate a stilbene-sensitive, electrogenic Na⁺-coupled HCO₃⁻ transporter in rat hippocampal neurons (603). Anoxia/ischemia or subsequent reoxygenation has also been shown to stimulate Na⁺-H⁺ exchange activity in mammalian neurons and astrocytes (43, 158, 276, 301, 497, 602), and such stimulation can involve kinases such as ERK1/2 and protein kinases A and C (301, 497, 602).
Effects of Cell Shrinkage on \( \text{pH}_i \)

**Effect of Hypertonic Solutions on \( \text{Na}^+ \)-\( \text{H}^+ \) and \( \text{Cl}^- \)-\( \text{HCO}_3^- \) Exchange**

In several cell types, including erythrocytes (92, 93, 314, 416, 417, 505, 506), lymphocytes (226), mesangial cells (44), glioma cells (273, 500), and barnacle muscle (140, 141), shrinking a cell in a hypertonic medium stimulates the exchange of external \( \text{Na}^+ \) for internal \( \text{H}^+ \). In at least *Amphiuma* red blood cells (RBCs), shrinkage in hypertonic solutions also stimulates the exchange of external \( \text{Cl}^- \) for internal \( \text{HCO}_3^- \). Because the \( \text{H}^+ \) extruded from the cells is derived almost exclusively from buffers, and because the \( \text{HCO}_3^- \) is derived from the freely diffusible \( \text{CO}_2 \) and \( \text{H}_2\text{O} \), these movements of \( \text{H}^+ \) and \( \text{HCO}_3^- \) are “osmotically silent.” Thus, the net effect of \( \text{Na}^+ \)-\( \text{H}^+ \) and \( \text{Cl}^- \)-\( \text{HCO}_3^- \) exchange is an uptake of \( \text{NaCl} \)—along with osmotically obligated \( \text{H}_2\text{O} \)—and a recovery of cell volume termed a “volume-regulatory increase” (VRI). As discussed in the following paragraphs, shrinkage appears to activate the \( \text{Na}^+ \)-\( \text{H}^+ \) exchanger directly. However, the stimulation of the \( \text{Cl}^- \)-\( \text{HCO}_3^- \) exchanger appears to be indirect, reflected by the rise in \( \text{pH}_i \) caused by the activated \( \text{Na}^+ \)-\( \text{H}^+ \) exchanger (264).

Interestingly, in lymphocytes, the shrinkage-induced activation of the \( \text{Na}^+ \)-\( \text{H}^+ \) exchanger is observed only after a volume-regulatory decrease that follows a previous cell swelling (226). Regulation of cell volume per se is discussed in Chapter 6. For other reviews of cell-volume regulation, see refs. (95, 229, 230, 243, 253, 312, 325, 326).

**Mechanism by Which Shrinkage/Hypertonicity Affects \( \text{Na}^+ \)-\( \text{H}^+ \) Exchange**

We do not know how cells sense shrinkage. However, we are beginning to understand more about the signal-transduction processes involved in the acute and chronic responses to shrinkage. Hypertonicity can trigger the rapid dephosphorylation phosphatidylinositol-5-kinase type I \( \beta \) (PIPKI\( \beta \)), which activates the enzyme and increases the rate of PIP2 synthesis (601). On a longer time scale, kinases in the Ste20 family, with the subsequent activation of the MAPK pathway, are critical for the chronic response to hypertonic shock in yeast (259, 302) and mammalian cells (495).

How does acute cell shrinkage affect one of the key endpoints of the signal-transduction cascade, namely, the \( \text{Na}^+ \)-\( \text{H}^+ \) exchanger? Although hypersmolarly does not alter NHE1 phosphorylation (231), the phosphorylation of other proteins is likely to be involved. For example, in work on primary rat astrocytes (501) and confluent C6 glioma cells (500), Shrode et al. have discovered that shrinkage appears to stimulate \( \text{Na}^+ \)-\( \text{H}^+ \) exchange through phosphorylation of myosin light chain. Kinases such as tyrosine kinases, MAPKs, and Janus kinase 2 (Jak2) also appear to be involved (11, 207, 214, 315). For example, the general tyrosine kinase inhibitor genistein inhibits tyrosine kinase-mediated protein phosphorylation and hypertonic-induced stimulation of NHE1 in polymorphonuclear leukocytes (315).

According to results from an intriguing structure-function study involving chimeras of NHE1 (stimulated by shrinkage) and NHE2 (not stimulated by shrinkage), the extracellular loop between TM domains 1 and 2 of NHE2 is sufficient to inhibit shrinkage-induced activation of the exchanger (533).

How does acute shrinkage mechanistically alter the \( \text{Na}^+ \)-\( \text{H}^+ \) exchanger? Dunham and colleagues (163, 164) have reported that cell shrinkage stimulates \( \text{Na}^+ \)-\( \text{H}^+ \) exchange activity in dog RBCs by reducing the transporter’s affinity for external \( \text{Na}^+ \) at an extracellular inhibitory site.

Other insights into the events linking shrinkage to the acute stimulation of \( \text{Na}^+ \)-\( \text{H}^+ \) exchange come from work on dog RBCs, lymphocytes, and/or giant-barnacle muscle fibers. First, although \( \text{Na}^+ \)-\( \text{H}^+ \) exchange appears inactive in these cells under physiological conditions, it can be stimulated by either a decrease in \( \text{pH}_i \) (139, 226), a decrease in cell volume (141, 226, 416), or an increase in \([\text{Li}^+]\) (139, 415). Second, the shrinkage-induced activation of \( \text{Na}^+ \)-\( \text{H}^+ \) exchange requires \( \text{Cl}^- \) (414, 416), specifically intracellular \( \text{Cl}^- \) (141, 258). In rat mesangial cells as well, the shrinkage-induced increase in \( \text{pH}_i \) is inhibited by preincubating the cells in a \( \text{Cl}^- \)-free solution for a minimum of 15 minutes (and presumably decreasing \([\text{Cl}^-]\), considerably) (44). Third, in barnacle muscle fibers, the shrinkage-induced activation of \( \text{Na}^+ \)-\( \text{H}^+ \) exchange is inhibited by GDP\( \beta \text{S}\) (140), suggesting involvement of a G protein in the signal transduction system. Consistent with this last observation, \( \text{Na}^+ \)-\( \text{H}^+ \) exchange is stimulated by either GTP\( \gamma \text{S}, \text{AlF}_3, \) or cholera toxin (CTX) (258). However, the CTX effect is not via a classic \( \text{G}_\text{i} \) pathway, inasmuch as neither cAMP nor cAMP analogues stimulate \( \text{Na}^+ \)-\( \text{H}^+ \) exchange. It should be emphasized that these data do not prove that CTX stimulates the \( \text{Na}^+ \)-\( \text{H}^+ \) exchanger by the same signal transduction pathway as does shrinkage. Finally, in barnacle muscle fibers, the \( \text{Cl}^- \) requirement in the shrinkage-induced activation of the exchanger is at or before activation of the heterotrimeric G protein (258).

**K\(^+\)-H\(^+\) Exchange**

Cala has observed that an exchange of internal K\(^+\) for external H\(^+\) contributes to the volume-regulatory decrease (VRD) that occurs after swelling *Amphiuma* RBCs (93). This exchange is inhibited in the nominal absence of Ca\(^2+\), and is stimulated by applying the Ca\(^2+\) ionophore A23187, suggesting that increases in \([\text{Ca}^{2+}]\), may activate K\(^+\)-H\(^+\) exchange. Two observations are of special interest. First, increased \([\text{Ca}^{2+}]\), failed to stimulate K\(^+\)-H\(^+\) exchange when Na\(^+\)-H\(^+\) exchange was stimulated by shrinkage. Second, if the cells were shrunk (so that Na\(^+\)-H\(^+\) exchange should have been stimulated) and then pretreated with amiloride (to block Na\(^+\)-H\(^+\) exchange), then swelling failed to activate K\(^+\)-H\(^+\) exchange. However, amiloride did not inhibit swelling-activate K\(^+\)-H\(^+\) exchange when the drug was applied for the first time to swollen cells. These data led Cala to suggest that Na\(^+\)-H\(^+\) and K\(^+\)-H\(^+\) exchange may be mediated by the same...
entity (94). K⁺–H⁺ exchange can also be activated by millimolar concentrations of N-ethylmaleimide (NEM) (5).

**Hormones, Chronic Stress, Growth Factors, and Oncogenes**

**HORMONES**

A number of hormones modulate transepithelial acid-base transport in various nephron segments (for examples, see [205, 265, 339, 410]). In addition, hormones modulate specific acid-base transporters in a variety of cells, including those from the kidney. The Na⁺–H⁺ exchanger is the transporter that has most often been the subject of these hormone studies—probably reflecting the transporter's popularity rather than its unique sensitivity to hormonal control. In the following paragraphs, we will review some of the hormones that can alter the activity of acid-base transporters, particularly those in the kidney.

**Glucocorticoids**

Elevations in plasma levels of glucocorticoids in response to metabolic acidosis increase acid excretion in the kidney by stimulating Na⁺–H⁺ exchange, thereby increasing H⁺ secretion and HCO₃⁻ reabsorption. In studies on brush-border membrane vesicles, Na⁺–H⁺ exchange rates are higher in vesicles obtained from adrenalectomized (adx) animals treated with the glucocorticoid dexamethasone than in adx animals treated with the mineralocorticoid aldosterone or adx animals receiving no supplementation (189, 299, 472). In proximal-tubule cells isolated from the kidney, Bidet et al. (51) have shown that even a 1-hour treatment with dexamethasone can enhance Na⁺–H⁺ exchange. The dexamethasone increases the Vₘₐₓ of the transporter, and stimulates new protein synthesis. Glucocorticoids exert their effect predominantly on the Na⁺–H⁺ exchange isoform NHE3. For example, in ileal brush-border membranes from animals injected with methylprednisolone, the glucocorticoid stimulates the activity and mRNA levels of NHE3, but not of NHE2 or NHE1 (609). In opossum kidney (OKP) cells, glucocorticoids increases both Na⁺–H⁺ exchange activity (35) and NHE3 mRNA levels (34). Hydrocortisone raises both the activity and protein level of NHE3 in OKP cells subjected to acidosis (20). More recent data are consistent with a nongenomic component to glucocorticoid-induced activation of NHE3 that involves both the stimulation of protein kinase SGK1 and the presence of the Na⁺–H⁺ exchanger regulatory factor NHERF2 (608). Glucocorticoids can also enhance the stimulatory effect of insulin on NHE3 activity and expression in OKP cells (195, 303).

Glucocorticoids appear to stimulate Na⁺/HCO₃⁻ co-transport as well. For example, removing hydrocortisone from the medium of cultured proximal tubule cells causes a decrease in Na⁺/HCO₃⁻ activity, whereas returning the hydrocortisone or adding dexamethasone increases the activity of the transporter (466). In addition, both NBCe1 activity and mRNA levels are increased 80% to 90% in proximal tubules from rats 4 days after subcutaneous injection with glucocorticoids (12).

Aldosterone can have both nongenomic (short term) and genomic effects (long term) on Na⁺–H⁺ exchange. For example, in renal and nonrenal cells, applying aldosterone elicits a stimulation of the Na⁺–H⁺ exchanger after a maximum delay of 20 minutes (400, 581–584), probably due to an alkali shift in the set point of the transporter (127). However, a 30-minute exposure of aldosterone to frog early distal tubule also stimulates Na⁺–H⁺ exchange activity, but due to increased expression of the transporter. In this frog-tubule preparation, the stimulation can be inhibited by transcription or translation inhibitors (128). In a nongenomic fashion, aldosterone also stimulates NHE3 activity in the mediullary thick ascending limb of the kidney (218, 219) through an ERK-dependent pathway (579). As presented in greater detail in Chapter 54, aldosterone also stimulates the V-type H⁺ pump in the distal tubule.

**Catecholamines**

The catecholamines norepinephrine and dopamine influence acid-base transporter activity in the kidney. Norepinephrine has been proposed to stimulate Na⁺–H⁺ exchanger activity in the proximal tubule (211, 399), probably through activation of α₃ and/or α₂ adrenergic receptors (338). However, in working on the *Ambystoma* proximal tubule, Abdulnour-Nakhoul et al. (2) found that norepinephrine has no effect on Na⁺–H⁺ exchanger activity, but rather, elicits an increase in pH, by inhibiting NBC-mediated HCO₃⁻ efflux.

Regarding dopamine, it is well established that this catecholamine inhibits Na⁺–H⁺ exchanger activity in the brush-border membrane of the proximal tubule (177, 178, 212, 269, 496, 594). Such inhibition is mediated through cAMP/ PKA-dependent and -independent events (177, 178, 594) and can involve endocytotic removal of NHE3 transporters from the apical membrane (262). Working on isolated proximal tubules from rabbit, Kunimi et al. (316) have demonstrated that dopamine also inhibits Na⁺/HCO₃⁻ co-transporter activity, although such inhibition was not observed in tubules from hypertensive rats. Dopamine has also been shown to inhibit Cl⁻–HCO₃⁻ exchanger activity in immortalized proximal tubule cells from normotensive, but not hypertensive rats (418).

Other agents that can influence acid-base activity in the kidney include adenosine, which stimulates Na⁺/HCO₃⁻ cotransporter activity in the proximal tubule (539), as well as cholinergic agonists that stimulate the cotransporter in proximal-tubule cells (451, 461).

**Thyroid Hormones**

The effect of thyroid hormone levels on Na⁺–H⁺ exchange activity has been examined in brush-border vesicles obtained from the renal cortex of hypothyroid, eu-, and hyperthyroid rats (297, 298, 300, 472). Compared with vesicles derived from euthyroid animals, those derived from hypothyroid animals had only half the normal Na⁺–H⁺ exchange activity, whereas those derived from hyperthyroid animals had twice the normal exchange activity. In both cases, the altered Na⁺–H⁺ exchange activity is due to an increase in the Vₘₐₓ of the transporter, rather than a decrease in the apparent Kₘ for Na⁺. The effect of thyroid hormones
triiodo-L-thyronine (T₃) and L-thyroxine (T₄) on Na⁺-H⁺ exchange has also been examined in cultured cells. In osmotic kidney cells, both T₃ and T₄ stimulate amiloride-sensitive ²²Na⁺ uptake (605), probably by increasing the transcription of NHE3 (97).

**Parathyroid Hormone, Angiotensin II, and Cyclic AMP**

In the renal proximal tubule, HCO₃⁻ reabsorption is inhibited by PTH (154, 234, 265), which increases intracellular levels of cAMP. At the level of brush-border membrane vesicles, PTH and cAMP analogues both reduce Na⁺-H⁺ exchange activity (278), whereas parathyroidectomy of the donor animal increases Na⁺-H⁺ exchange activity (123). At the cellular level, PTH and cAMP both inhibit Na⁺-H⁺ exchange in a renal epithelial cell line (383, 423) and in rat medullary thick ascending limb (mTAL) tubule suspensions (54).

Based on work by Weinman and colleagues (586), the inhibitory effect of cAMP in the proximal tubule requires the presence of the Na⁺-H⁺ exchange regulatory factory NHERF1, which is a PDZ adaptor protein that can bind to the carboxy terminus of NHE3 (588, 589). The outcome of PTH stimulation is probably a shift in the pH₄ sensitivity of Na⁺-H⁺ exchange (368), likely associated with PTH-induced phosphorylation of NHE3 (124, 173). PTH can also have a delayed secondary effect of stimulating internalization of NHE3 from apical membranes (124, 173, 613).

PTH and cAMP can also inhibit HCO₃⁻ transporters. Treatments that raise [cAMP] inhibit apical Cl⁻-HCO₃⁻ exchange in the Necturus gallbladder epithelium (445) and in renal epithelial cells (245). Similarly PTH appears to inhibit Na⁺+/HCO₃⁻ cotransport activity in rabbit renal basolateral membranes via G proteins and a calmodulin-dependent protein kinase (462). Increases in cAMP and activated Ca²⁺-dependent protein kinases also inhibit Na⁺+/HCO₃⁻ cotransport in these basolateral membranes (460). As described previously for cAMP-mediated inhibition of NHE3, NHERF1 is also involved in cAMP-mediated inhibition of NBCe1, although there is no direct association between the two proteins (41, 587).

Low doses of angiotensin (Ang) II (e.g., 10⁻⁹ M) stimulate Na⁺-H⁺ exchange and/or Na⁺+/HCO₃⁻ cotransport in the proximal tubule (129, 167, 209, 246, 248, 261, 339, 339, 442, 471, 616), distal tubule (33, 332, 577), and cortical collecting duct (482). On the other hand, higher doses of Ang II (e.g., 10⁻⁶ M) inhibit these transporters (37, 129, 261, 616). Long-term effects of Ang II include regulation of NBCe1 expression in the proximal tubule. For example, expression levels of NBCe1—but not NHE3—in the kidney increase when rats are infused with Ang II, but decrease when infused with the AT1 receptor blocker candesartan (560). Regarding the signal–transduction pathway, in renal cortical basolateral membrane vesicles (464) and OKP cells (98), Ang II stimulation of the exchanger involves G-protein activation. Ang II stimulation of Na⁺+/HCO₃⁻ cotransporter activity in OKP cells involves activation of Src family tyrosine kinases (SFKs) and the classic MAPK pathway (452).

In isolated perfused superficial S1 segments of the rabbit proximal tubule, Ang II stimulates Na⁺-H⁺ exchange and Na⁺+/HCO₃⁻ cotransport (209), as judged from rates of pH₄ change measured at relatively low pH₄ values. Interestingly, Ang II has very little effect on steady-state pH₄. Thus, if Ang II indeed stimulates both transporters in the physiological pH₄ range, then the combined increase in activities of the acid exchanger and acid loader serves to increase transepithelial HCO₃⁻ reabsorption without altering pH₄. Ang II can also stimulate the Na⁺-H⁺ exchangers on the apical (419) and basolateral (37) sides of macula densa cells.

Ang II can also stimulate other acid-base transporters. For example, in cat papillary muscles of the ventricular myocardium, Ang II stimulates Cl⁻-HCO₃⁻ exchange, via activation of a PKC-dependent pathway (96). In the kidney, Ang II has been shown to stimulate the H⁺ pump in isolated proximal tubule cells by colchicine-sensitive apical membrane insertion (572).

**Chronic Stresses**

**Chronic Metabolic Acidosis and Alkalosis**

Chronic metabolic acidosis typically increases Na⁺-H⁺ exchange activity in the kidney, whereas chronic metabolic alkalosis has the opposite effect. For example, brush-border membrane vesicles derived from animals with chronic metabolic acidosis have increased Na⁺-H⁺ exchange activity (123), due to an increase in the apparent Vₘₐₓ, rather than to a decrease in the Kₘ for Na⁺ (293, 472). Increases in the activity of Na⁺-H⁺ exchange have been observed in other preparations, such as membrane vesicles of rabbit kidney proximal tubules previously incubated in a low-pH solution (521), and cells from the medullary thick ascending limb (mTAL) of rats subjected to metabolic acidosis (323). In the mTAL, such activation is paralleled by increases in NHE3 mRNA and protein levels (323), but not NHE1 mRNA levels (324). Working with cultured proximal-tubule cells, Alpern et al. (260) found that chronic metabolic and respiratory acidosis increases Na⁺-H⁺ exchange activity via new protein synthesis. This effect, as well as increased expression of NHE3 mRNA, is blocked by overexpressing Csk (600), a natural inhibitor of Src kinases. Recall that in acute experiments (see previous sections) on isolated perfused proximal tubules, CO₂ appears to signal through a receptor tyrosine kinase, not Src.

Chronic metabolic acidosis/alkalosis can also elicit changes in the activity of other acid-base transporters. For example, the increase in apical Na⁺-H⁺ exchange activity with metabolic acidosis is paralleled by an increase in basolateral Na⁺+/HCO₃⁻ cotransporter activity (10, 428, 521). More recently, Kwon et al. (320) used semiquantitative immunoblotting and immunohistochemistry to examine the effect of chronic metabolic acidosis on the expression of three Na⁺-coupled HCO₃⁻ transporters in rat kidney. The acidosis increased NBCn1 abundance in the medullary thick ascending limb and another NBCn1 variant in intercalated
cells, but had no effect on NBCe1 abundance in the proximal tubule.

Metabolic acidosis also causes an increase in the activity of apical H\(^+\) pumps in the kidney (522, 558, 559), presumably contributing to the increase in HCO\(_3^-\) reabsorption. In fact, chronic acidosis stimulates the redistribution of H\(^+\) pumps to the apical surface of collecting-duct intercalated cells, whereas chronic alkalosis stimulates the redistribution to the basolateral surface (470). Finally, Sabolic et al. (470) observed that expression levels of Cl\(^-\)/HCO\(_3^-\) exchange increase in the cortical collecting duct (CCD) of rats subjected to chronic metabolic acidosis, but decrease in animals subjected to chronic metabolic alkalosis. Also, working on the CCD, Tsuruoka and Schwartz (558) found that chronic metabolic acidosis decreases apical Cl\(^-\)/HCO\(_3^-\) exchange activity (249, 491). Interestingly, the denervation associated with the uninephrectomy may be the predominant stimulus.

Indeed, the hypertrophy-induced activation of Na\(^+/\)H\(^+\) exchange (249, 491) involves activation of Src family kinases and phosphatidylinositol 3 kinase and increased membrane insertion of NBCe1 (40, 171, 463, 465). Later (24 hours), these authors observe increases in mRNA and protein levels. In isolated perfused proximal tubules, an inhibitor of Src kinases has no effect on NBCe1 activity that initially (within 5 minutes) involves activation of Src family kinases and phosphatidylinositol 3 kinase and increased membrane insertion of NBCe1 (540). Na\(^+/\)H\(^+\) exchange activity is increased in renal brush-border vesicles derived from animals that were chronically hypercapnic (611). Chronic respiratory acidosis also increases levels of band 3 mRNA in the kidney (540). Na\(^+/\)H\(^+\) exchange activity is increased in renal brush-border vesicles derived from animals that were chronically hypercapnic (611). Chronic respiratory acidosis also increases levels of band 3 mRNA in the kidney (540). Working on either opossum kidney cells or cultures of proximal-tubule cells, Arruda and colleagues have found that 10% CO\(_2\) (i.e., respiratory acidosis) has a biphasic stimulatory effect on NBCe1 activity that initially (within 5 minutes) involves activation of Src family kinases and phosphatidylinositol 3 kinase and increased membrane insertion of NBCe1 (40, 171, 463, 465). Later (24 hours), these authors observe increases in mRNA and protein levels. In isolated perfused proximal tubules, an inhibitor of Src kinases has no effect on the acute stimulation of HCO\(_3^-\) reabsorption by increased levels of basolateral CO\(_2\) (617).

**Renal Hypertrophy, High-Protein Diet, and Chronic Potassium Depletion** Renal hypertrophy can increase the activity of several acid-base transporters (99). Brush-border membrane vesicles derived from the remnant kidney following a uninephrectomy exhibit increased Na\(^+/\)H\(^+\) exchange activity (249, 491). Interestingly, the denervation associated with the uninephrectomy may be the predominant stimulus. Indeed, the hypertrophy-induced activation of Na\(^+/\)H\(^+\) exchange elicited by the uninephrectomy can be mimicked by contralateral denervation (347). Nevertheless, hypertrophy of renal proximal-tubules cell in vitro does lead to an increase in Na\(^+/\)H\(^+\) exchange activity (182). In microperfusion studies of rat proximal tubule, Preisig and Alpert (429) demonstrated that the hyperfiltration preceding a decrease in renal mass increases the activities of Na\(^+/\)H\(^+\) exchange and Na\(^+/\)HCO\(_3^-\) cotransport.

Rats fed a 40% protein diet, rather than a 6% protein diet, also yield cortical brush-border membrane vesicles with enhanced Na\(^+/\)H\(^+\) exchange activity, and the effects of uninephrectomy and high-protein diet are additive (249, 491).

Finally, K\(^+\) depletion can also increase the function and expression of acid-base transporters. For example, vesicles derived from K\(^+\)-depleted rats exhibit enhanced Na\(^+/\)H\(^+\) exchange and Na\(^+/\)HCO\(_3^-\) cotransport activity due to increases in apparent V\(_{\text{max}}\) values, with no changes in the apparent K\(_{\text{m}}\) values for Na\(^+\) (520). Also, in functional and antibody studies, dietary K\(^+\) depletion enhances the H\(^+\) pump in rat distal nephron (30). As reviewed by Silver and Soleimani (510), K\(^+\) depletion also increases function and expression of K\(^+/\)H\(^+\) pumps in the kidney, particularly the colonic form of the K\(^+/\)H\(^+\) pump. K\(^+\) depletion appears to enhance overall HCO\(_3^-\) reabsorption in the renal tubule by increasing NBCe1 expression and activity in the proximal tubule, as well as eliciting NBCe1 mRNA expression and/or transporter activity in the mTAL and inner medullary collecting duct (21).

**Growth Factors**

*Classical Model of Growth-Factor Effects on pH. A potential role for pH\(_i\) in mitogenesis is suggested by the observation that growth-factor–induced proliferation of fibroblasts (425) and mesangial cells (202) is blocked at relatively low pH\(_i\) values, and rises sharply as pH\(_i\) enters the physiological range. Indeed, there are numerous examples in which adding a mitogen to quiescent cells, in the nominal absence of CO\(_2\)/HCO\(_3^-\), causes a sustained increase in pH\(_i\) (199, 227, 380, 432, 458, 483, 532, 538, 567). Because the mitogen–induced pH\(_i\) increase is prevented by removing Na\(^+\) (227, 380) or pretreating with amiloride or an amiloride analogue (227, 322, 380), many investigators had deduced that the increase in pH\(_i\) is due to stimulation of Na\(^+/\)H\(^+\) exchange (for more examples, see review [377]). Furthermore, in fibroblast mutants lacking Na\(^+/\)H\(^+\) exchange, mitogens fail to elicit proliferation, or to alkalinate the cells (425, 426). Wang et al. (576) have reported that a P19 embryonal carcinoma cell line lacking Na\(^+/\)H\(^+\) exchange has a reduced ability to grow and differentiate. The ability to differentiate can be resurrected by reintroducing the exchange into the cells. These data led to an attractive hypothesis: (1) in quiescent cells, pH\(_i\) is too low to sustain proliferation; (2) mitogens, in addition to affecting other cellular functions, stimulate Na\(^+/\)H\(^+\) exchange; (3) this stimulation of Na\(^+/\)H\(^+\) exchange elevates pH\(_i\) to a range that is permissive for proliferation. Thus, according to this model, pH\(_i\) would play a central role in proliferation.*
(202). In all four of the aforementioned cell types, the steady-state pH$_i$ in the presence of CO$_2$/HCO$_3$ is substantially higher than that observed in the absence of CO$_2$/HCO$_3$, with or without mitogen. Thus, it seems that, at least for some cells, the pH$_i$ prevailing in the presence of CO$_2$/HCO$_3$ is high enough to put pH$_i$ in a range permissive for proliferation, and that pH$_i$ remains in this permissive range even after a mitogen-induced acidification. Although the pH$_i$ increase elicited by mitogens in the absence of CO$_2$/HCO$_3$ is real, it appears that a change in pH$_i$ is not an intrinsic part of the mitogenic response.

Transports Affected by Mitogens in the Presence of CO$_2$/HCO$_3$ The effect of growth factors on the activity of acid-base transporters of cells incubated in the presence of CO$_2$/HCO$_3$ has been examined in some preparations. In renal mesangial cells, application of arginine vasopressin (AVP) (198, 200) or epidermal growth factor (EGF) (198) “activates” all three acid-base transporters known to be present: the Na$^+$/H$^+$ exchanger, the Na$^+$/driven Cl$^-$-HCO$_3$ exchanger and the Cl$^-$-HCO$_3$ exchanger. The two acid-extrusion mechanisms, assayed at the single pH$_i$ of 6.6, were stimulated by ~100%. The acid loader, assayed at the single pH$_i$ of 7.7, was stimulated to an even greater extent, ~140%. AVP also stimulates both Na$^+$/H$^+$ and Cl$^-$-HCO$_3$ exchange in the A10 vascular-smooth-muscle cell line (290). It should be emphasized that none of the data discussed thus far address the issue of whether in fact the growth factors stimulated the transporters in the physiological pH$_i$ range (i.e., approximately 7.1–7.3 for these cells).

Growth factors can also have time-dependent effects on pH$_i$ and acid-base transporters. For example, in working on mesangial cells, Ganz et al. (198) observed that both AVP and EGF, which are thought to act through different signal transduction pathways, have similar effects on the time courses of the three aforementioned transporters. Both growth factors elicit an immediate (i.e., within 10 minutes) increase in the activities of all three transporters (assayed at the single pH$_i$ values indicated previously). The immediate response is followed within an hour by a fall in the activities of all three transporters, even though the activities remain substantially above control levels. Finally, there is a transient dip in the activities of all three transporters at times corresponding to the period of the maximal rate of increase in cell number (~35–45 hours for AVP and ~12–20 hours for EGF). Growth factors can have “early” and “late” effects on Na$^+$/H$^+$ exchange in vascular smooth muscle cells (see review [343]). The late effect can include increased expression of the exchanger.

Effect of Growth Factors on the pH$_i$ Dependence of the Na$^+$/H$^+$ Exchanger In the past, many investigators believed that the pH$_i$ dependence of the Na$^+$/H$^+$ exchanger is linear, and that the exchanger is inactive at the physiological (i.e., “threshold”) pH$_i$. Investigators also believed that the mitogen-induced increase in pH$_i$ that is observed in the absence of CO$_2$/HCO$_3$ exclusively reflects stimulation of Na$^+$/H$^+$ exchange in the physiological pH$_i$ range. According to this view, mitogens cause an alkaline shift and/or an increase in the steepness of the Na$^+$/H$^+$ exchange activity versus pH$_i$ relationship, without having other effects on acid extrusion and/or acid loading. Indeed, careful analyses of the pH$_i$ dependence of Na$^+$/H$^+$ exchanger activity in intact hepatocytes indicates that Na$^+$/H$^+$ exchange activity can vary linearly with pH$_i$ (82, 530) and that a mitogen can shift this line in the alkaline direction (530). However, as discussed previously (pH$_i$ dependence of Na$^+$/H$^+$ exchange), it is clear that the approach often used to reach similar conclusions in other cells (i.e., comparison of pH$_i$ recovery rates with and without amiloride at a single low pH$_i$) is seriously flawed.

Applying the approach used to generate the pH$_i$ dependence of Na$^+$/H$^+$ exchange activity in Fig. 11A or Fig. 11B leads to the conclusion that AVP has a complex pH$_i$- and time-dependent effect on Na$^+$/H$^+$ exchange in mesangial cells. At short times (~8 minutes) after applying AVP, the pH$_i$ dependence of Na$^+$/H$^+$ exchange activity (similar to that shown in Fig. 14A) becomes linear in such a way that the transporter is unaffected at pH$_i$ values below 6.7, but inhibited at higher pH$_i$ values. At longer times (~14 minutes), the new linearized plot is shifted to more alkaline values. In the new “stimulated” steady state, the Na$^+$/H$^+$ exchanger is more active at pH$_i$ values below ~6.9, but less active at higher pH$_i$ values. How can AVP therefore raise pH$_i$ in the absence of CO$_2$/HCO$_3$, if the Na$^+$/H$^+$ exchanger is inhibited in the physiological pH$_i$ range? The answer seems to be that AVP has an even greater inhibitory effect on background acid-loading processes. Thus, AVP causes pH$_i$ to rise in mesangial cells in the absence of CO$_2$/HCO$_3$ not because the growth factor stimulates Na$^+$/H$^+$ exchange, but because it inhibits Na$^+$/H$^+$ exchange to a lesser extent than it inhibits background acid loading.

Oncogenes

Hagag et al. (238) found that microinjecting the v-H-ras p21 gene product into mouse NIH 3T3 cells causes a rapid and sustained pH$_i$ increase that is inhibited by amiloride or low [Na$^+$]. Thus, a functional Na$^+$/H$^+$ exchanger is required for the ras-induced pH$_i$ increase, although these data do not prove that ras actually stimulates the exchanger. Doppler et al. (160) took a different approach, transfecting NIH 3T3 cells with the v-mos or Ha-ras oncogene under the control of the MMTV-LTR promoter. They found that expression of either oncogene, upon addition of a glucocorticoid, elicited an increase in pH$_i$, as well as progression into the S phase of the cell cycle. Expression of the proto-oncogene of Ha-ras (i.e., the normal cell product) had no affect on pH$_i$, and was only weakly mitogenic. The pH$_i$ increase associated with expression of the Ha-ras oncogene was blocked by dimethylamiloride (352), demonstrating a requirement for a functional Na$^+$/H$^+$ exchanger. In NIH


3T3 cells transformed with the c-H-ras oncogene, Kaplan and Boron (285) found that a higher steady-state pH_i — compared to that of the nontransformed cells—was due to an alkali shift of ~0.7 pH unit in the pH_i dependencies of both the Na+/H+ exchanger and the Na+-driven Cl-/HCO_3^- exchanger.

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