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Topical Review

Transport of H^+ and of Ionic Weak Acids and Bases

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I. Introduction

The transport of H^+ and of various ionic weak acids and bases (e.g., NH_4^+ and HCO_3^-) is known to be of great importance for several cellular organelles, for individual cells, and for epithelia. The purpose of this Topical Review is to examine some aspects of the transport of these substances across the plasma membrane of animal cells. The transport of H^+ and ionic weak acids and bases can be divided into two broad categories: passive and active processes. The passive processes, such as H^+ influx and HCO_3^- efflux, generally impose a chronic acid load on the cell's interior. If the acidifying effects of such passive fluxes are not counteracted by an active transport process, intracellular pH (pH_i) would gradually fall to dangerously low levels. The active processes fall in two categories: In the case of "primary" active transport, the energy for moving a solute is derived directly from the hydrolysis of ATP or from electron transport. In the case of "secondary" active transport, the energy for moving a solute is provided by coupling its movement to that of another solute down the latter's electrochemical gradient. The primary active transport of H^+ is known to occur in certain "tight" epithelia which generate sizeable transepithelial pH gradients. It is not known whether such primary active transport mechanisms, or "pumps," contribute to pH_i regulation in these epithelia. Inasmuch as these pumps have been the subject of several recent reviews, the former will receive only cursory attention here. The secondary active transport of H^+ or HCO_3^- is known to occur in nerve and muscle, and in certain "leaky" epithelial cells. These transporters counteract the acidifying effects of passive ion fluxes, and are highly efficient regulators of pH_i. In certain leaky epithelia, they are responsible for transepithelial acid transport.

For a recent, comprehensive summary of the literature on intracellular pH, the reader is referred to the review by Roos and Boron (1981).

II. Passive Transport Processes

A. H^+/OH^-

There is no certain basis for distinguishing the transport of H^+ from that of OH^- in the opposite direction. Accordingly, I shall refer to H^+ transport with the understanding that the transport process in question might well involve OH^- instead of, or in addition to, H^+. Two general approaches have been used to provide insight into membrane permeability to H^+ (P_H). One is illustrated by the work of Izutsu (1972), who exposed frog skeletal muscle to a relatively acidic, HCO_3^--containing external solution, while using the distribution of the weak acid 5,5-dimethyl-2,4-oxazoladinedione (DMO) to periodically measure the resultant fall in pH_i. From the rate of decline of pH_i, he calculated that P_H would have to be about three orders of magnitude greater than P_K in order for the fall in pH_i to be explained solely by the passive influx of H^+. On the other hand, P_{HCO_3} would have to be only about two orders of magnitude less than P_{Cl} in order for the observed intracellular acidification to be accounted for solely by passive HCO_3^- efflux. This approach for estimating P_H requires that no other processes (e.g., lactic acid production) contribute to the decline in pH_i, and obviously cannot distinguish between fluxes of H^+ and HCO_3^- if the latter ion is present. A related problem is that reductions in extracellular pH (pH_o) generally inhibit active transport pro-

cesses responsible for the regulation of pH_i in the alkaline direction. Thus, it may be difficult to determine how much of the fall in pH_i is due to increased H^+ influx, and how much to decreased active transport. Another approach for estimating P_H, used by Woodbury in 1971, involves measuring changes in membrane potential and resistance while altering pH_o. By comparing the effects of altering pH_o with those produced by altering $[Cl^-]_o$, one can theoretically obtain P_H/P_{Cl} (in HCO_3^--free solutions) or P_{HCO_3}/P_{Cl} (in HCO_3^--containing solutions). Woodbury obtained values of ~ 500 for P_H/P_{Cl}, and ~ 0.1 for P_{HCO_3}/P_{Cl}. This approach is greatly complicated, however, by the likelihood that changes in pH_o directly affect membrane potential and/or resistance by altering membrane permeability to ions (e.g., K^+) other than H^+ or HCO_3^-.

Given the relatively high mobility of H^+ in liquid water (~ 5 times that of K^+), there is good reason to expect P_H to be relatively high in biological membranes. The aforementioned difficulties in calculating P_H from changes in either pH_i or V_m preclude placing a precise value on P_H at present, at least for intact cells. Several investigators (for review, *see* Deamer, 1982) have studied H^+ permeability in systems of artificial lipid bilayers, obtaining values of about 10^{-3} to 10^{-9} cm/sec for P_H. These data provide an estimate of H^+ permeability through the hydrophobic region of cell membrane (for comparison, P_K of intact cells is generally 10^{-6} to 10^{-7} cm/sec), but do not address the possibility of H^+ permeation through or around membrane proteins. However, even if P_H were reasonably high in cell membranes, effects of passive H^+ fluxes on pH_i would be expected to be slight in the short term. This is because values of $[H^+]$ are normally so low that net H^+ fluxes are expected to be small. In the longer term, however, the net, passive influx of H^+ could represent a substantial, albeit slowly developing, intracellular acid load which must be counteracted by active processes.

B. HCO_3^- and Other Anionic Weak Bases

There are considerable data in muscle which are consistent with permeability of the plasma membrane to HCO_3^-. As we shall see, however, these data are by no means conclusive proof that HCO_3^- can traverse the membrane by a purely diffusive route. The main evidence which one might muster in support of passive HCO_3^- permeability comes from several groups of workers (Adler, Roy & Relman, 1965*a, b;* Izutsu, 1972; Aickin & Thomas, 1975, 1977*a*; Gonzalez & Clancy, 1975; Ellis & Thomas, 1976; Khuri, Agulian & Bogharian, 1976; Bolton & Vaughan-Jones, 1977; Boron, 1977) who have found that muscles exposed to a low-pH, low-$[HCO_3^-]$ solution (so that an outwardly directed electrochemical gradient for HCO_3^- is established) exhibit a slow fall in pH_i. This observation, made using both pH-sensitive microelectrodes and DMO to measure pH_i, suggests that the observed intracellular acidification may have been due to the net, passive efflux of HCO_3^-. In these studies, however, factors other than the passive exit of HCO_3^- could have contributed to the observed fall in pH_i. As noted above, for example, lowering pH_o inhibits the active processes which normally serve to raise pH_i, so that pH_i would be expected to decline independently of any direct effect on passive HCO_3^- efflux. Other data (*see* section *IIIA1,* below) suggest that when pH_o is lowered sufficiently, the active processes may actually be *reversed,* and this could also contribute to the observed decline in pH_i. Thus, there is as yet no convincing evidence of a significant diffusive pathway for HCO_3^- in muscle.

The data are more convincing for permeability to HCO_3^- (or to related species, such as CO_3^{2-}) across the basolateral (i.e., blood-side) membrane of renal proximal tubule cells. Frömter and his colleagues (Frömter, Sato & Gessner, 1975; Burckhardt & Frömter, 1980), working on *in vivo* rat proximal tubules, have shown that suddenly reducing $[HCO_3^-]$ in the external solution at the basolateral or blood-side surface of the cells ($[HCO_3^-]_{bl}$) causes rapid but transient depolarization of the basolateral membrane. The effect is reversed when $[HCO_3^-]_{bl}$ is returned to its initial level, and all the voltage (V_{bl}) changes are inhibited by carbonic anhydrase inhibitors and by the stilbene derivative 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS). These results were interpreted as supporting the hypothesis of basolateral permeability either to HCO_3^- or to OH^- (which would be indistinguishable from H^+ permeability). Experiments on isolated, perfused salamander proximal tubules (Boron & Boulpaep, 1982) have confirmed that simultaneously reducing $[HCO_3^-]_{bl}$ and pH_{bl} (at constant pCO_2) causes a transient basolateral depolarization. In addition, simultaneous measurements of pH_i (with pH-sensitive microelectrodes) showed that this reduction of $[HCO_3^-]_{bl}$ and pH_{bl} is accompanied by the rapid, SITS-sensitive fall of pH_i. Inasmuch as these changes in pH_i and V_{bl} were substantially reduced in the nominal absence of HCO_3^-, the authors concluded that the movement of HCO_3^- (or of a related species) was involved. Further work by these authors on the same

preparation strongly suggests that basolateral transport of HCO_3^- may be tightly coupled to the movement of Na^+ in the same direction: (i) reducing $[Na^+]_{bl}$ causes the same pattern of changes in V_{bl} and pH_i as does reducing $[HCO_3^-]_{bl}$, (ii) these effects of lowering $[Na^+]_{bl}$ are blocked by SITS, and (iii) the reduction of $[HCO_3^-]_{bl}$ causes the transient, SITS-sensitive fall of intracellular Na^+ activity. The movement of HCO_3^- (or a related species) may be tightly coupled to that of Na^+ across the basolateral membrane in such a way that the net process carries net negative charge. Possible mechanisms of transport include the movement of two HCO_3^- plus one Na^+, one CO_3^{2-} plus one Na^+, and the transport of a single $NaCO_3^-$ ion pair. It is unclear whether the mechanism of transport is a simple diffusive process (as could be the case for $NaCO_3^-$), or whether carrier-mediated transport is involved (as would have to be the case for the other two models). Since this Na-coupled HCO_3^- transport is easily reversed, the reaction is probably driven solely by ion gradients, so that it can be regarded as a "secondary" active process.

There is also evidence for a diffusive permeability of the cell membrane to certain other anions (A^-) of neutral weak acids (HA). When a cell is exposed to a solution containing both A^- and HA, pH_i first falls relatively rapidly as the neutral acid enters and subsequently dissociates into A^- and H^+. This fall in pH_i, however, gradually slows as $[HA]_i$ approaches $[HA]_o$. Once $[HA]_i$ equals $[HA]_o$, there is no longer net entry of HA and, therefore, no further fall of pH_i by this route. The subsequent course of pH_i in such an experiment depends critically on the interplay between (i) active transport processes which are stimulated by the decrease in pH_i and which tend to return pH_i toward normal and (ii) the passive efflux of A^-, which tends to lower pH_i. Inasmuch as subsequent changes in pH_i are relatively slow, this period is often termed the "plateau phase." We can describe three general patterns of pH_i changes during the plateau phase (depicted in the idealized experiments of Fig. 1). In the first, the rate of acid extrusion exceeds the passive efflux of A^-. Here, pH_i should gradually return toward its initial value after the initial HA-induced fall in pH_i (Fig. 1, left). If the permeability to A^- (P_A) is zero, pH_i will return to its initial value; if P_A is nonzero, then the new steady-state pH_i will be less than the initial value. An example is the snail neuron exposed to the weak acid CO_2 at constant pH_o (Thomas, 1976*a*). There is a rapid fall in pH_i (complete in ~2–3 min), due to the entry of CO_2, followed by a slower, exponential

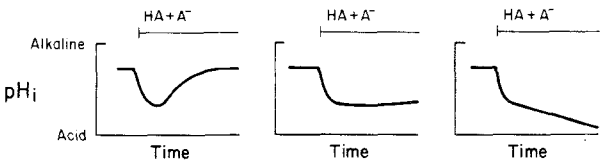

Fig. 1. Model experiments depicting the response of intracellular pH (pH_i) to the application of a neutral weak acid (HA) and its conjugate weak base (A^-). In all three examples application of HA plus A^- initially causes an abrupt fall in pH_i due to the rapid influx of the neutral weak acid. *A*. The passive efflux of A^- is zero, and the cell's pH_i-regulating system is able to return pH_i to normal after the initial acidification. *B*. The passive efflux of A^- exactly balances acid extrusion, and pH_i stabilizes after the initial acidification. *C*. The passive efflux of A^- exceeds the acid-extruding capacity of the cell, and pH_i continues to fall after the rapid, initial acidification. Note that pH is plotted according to the American Convention, with high pH values at the top

return of pH_i (rate constant, ~5 min) to its normal value, due to an active transport process. The second pattern occurs when the rate of acid extrusion is just matched by the passive efflux of A^-. Here, an exposure to the weak acid should cause a fall in pH_i from which there is no recovery (Fig. 1, center). An example of this is found in the salamander's renal proximal tubule cell (Boron & Boulpaep, 1982). Exposure to CO_2 causes a rapid and sustained fall in pH_i since a substantial efflux of HCO_3^- across the cell's basolateral membrane matches the efforts of the cell's active, acid-extruding mechanism. The third pattern of plateau-phase pH_i changes occurs when A^- efflux exceeds acid extrusion. In this case, pH_i falls rapidly, due to the influx of HA, and then continues to fall more slowly, due to the passive efflux of A^- (Fig. 1, right). Such a situation could develop if the acid-extruding process were inhibited or if permeability to A^- were substantial. An example of the former is illustrated by an experiment of Keifer and Roos (1981), who exposed a barnacle muscle fiber to the weak acid DMO while simultaneously blocking acid extrusion by lowering pH_o. This exposure to DMO caused pH_i fo fall in two distinct phases: a rapid one (due to influx of HA), and a slower one (due to the uncompensated-for efflux of A^-). Their data led them to conclude that, in the case of DMO, the cell membrane is about 0.1% as permeable to A^- as to HA. Another example of this general phenomenon is an experiment of Sharp and Thomas (1981), who exposed crab muscle fibers to salicylic acid. After the initial, rapid fall of pH_i due to the entry of the neutral weak acid, pH_i continued to fall at a somewhat slower rate, reflecting a substantial permeability to the salicylate anion. Similar experiments provided evidence

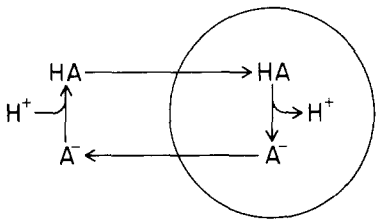

Fig. 2. Schematic representation of the passive efflux of A^- and how this leads to the passive influx of HA. The counterfluxes of A^- and HA serve as a shuttle, carrying H^+ into the cell

for significant permeabilities to formate and propionate. Estimates of the extra intracellular buffering power provided by propionic acid/propionate, as well as direct measurements of $[propionate]_i$ with a Cl-sensitive liquid ion-exchange electrode (which also has a substantial sensitivity to propionate), led Sharp and Thomas to conclude that during the plateau phase, $[propionic\ acid]_i$ is only 25 to 50% as high as $[propionic\ acid]_o$. As can be seen from Fig. 2, this is exactly what would be expected if a large efflux of A^- shifted the intracellular equilibrium $HA = H^+ + A^-$ to the right, thereby reducing $[HA]_i$ below $[HA]_o$. This, in turn, leads to the influx of HA. The countermovements of HA and A^- act as a shuttling system, effectively carrying protons into the cell along their electrochemical gradient (Boron & DeWeer, 1976*a*). That $[HA]_i$ should be less than $[HA]_o$ had previously been predicted (*see* Roos & Boron, 1981), but never directly demonstrated. The simultaneous movements of HA and A^-, and their effects of pH_i, were mathematically modeled by Boron and DeWeer, (1976*a*), and subsequently refined by Keifer and Roos (1981).

C. NH_4^+ and Other Cationic Weak Acids

It has been known for over 60 years (Jacobs, 1922–1923) that exposing a cell to an NH_3-containing solution causes pH_i to rise. This is not surprising, inasmuch as the cell membrane is expected to be far more permeable to the neutral weak base NH_3 than to the charged, conjugate weak acid, NH_4^+. In the past few years, however, it has become clear that permeation by NH_4^+ can also have a significant, though smaller, effect on pH_i. The fundamental observation (*see* Fig. 3*A*) is that exposing a cell to a solution containing NH_3 causes a rapid rise in pH_i, followed by a much slower fall (Boron & DeWeer, 1976*a*). The rapid alkalinization (segment *ab*) is due to the influx of NH_3, followed by its combination with H^+ to yield NH_4^+ intracellularly. This tendency toward intracellular

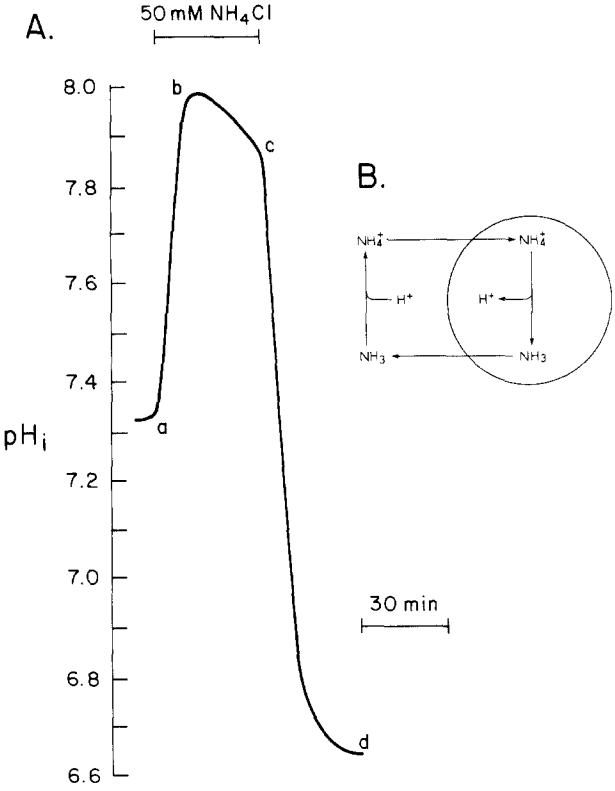

Fig. 3. *A.* An experiment on a squid giant axon illustrating the effect of exposing a cell to a solution containing NH_4^+ and its conjugate weak base, NH_3. Segment *ab* is dominated by the net influx of NH_3, segment *bc* by the net influx of NH_4^+, and segment *cd* by the washout of NH_3. *B.* Schematic representation of the passive influx of NH_4^+ during segment *bc* above. This influx leads to the efflux of NH_3. Thus, the counterfluxes of NH_4^+ and NH_3 serve as a shuttle, carrying H^+ into the cell

alkalinization wanes as $[NH_3]_i$ approaches $[NH_3]_o$, and the net influx of NH_3 declines. Even as the entry of NH_3 is causing pH_i to rise, however, the much slower entry of NH_4^+ is having the opposite effect. The acidifying tendency of NH_4^+ influx becomes evident only after NH_3 entry has slowed sufficiently. The resultant slow downward drift of pH_i, during a period termed the "plateau phase" (*bc*), appears to be due primarily to the passive entry of NH_4^+ (*see* Fig. 3*B*). Indeed, reversal of the NH_4^+ electrochemical gradient actually leads to a plateau-phase alkalinization (Boron, 1977). When mouse skeletal muscle is exposed to a K^+-free Ringer's, NH_4^+ can also be carried into the cell by the Na–K pump, which apparently transports NH_4^+ in the absence of external K^+ (Aickin & Thomas, 1977*a*).

One reason why permeability to NH_4^+ is of considerable practical importance becomes apparent when a cell is returned to an NH_4^+/NH_3-free solution: virtually all intracellular NH_4^+, including that

which previously had entered directly from the outside, gives up a H^+ and exits the cell as NH_3. As a result, pH_i falls far below its initial value (*cd*), this undershoot directly reflecting the previous net entry of NH_4^+. The practical importance of this is that the undershoot represents an intracellular acid load which greatly stimulates the active, acid-extruding processes discussed in detail below. An exposure to NH_4^+ thus provides a clean method for acid-loading cells; it offers the advantage that at the time when pH_i is minimal, virtually all NH_4^+ and NH_3 is removed from the system. This approach has been employed in a variety of cell types (*see* Roos & Boron, 1981).

In principal, any cationic weak acid should behave similarly to NH_4^+. At present the only such evidence is for methylammonium (Boron & Roos, 1976). The simultaneous movements of NH_3 and NH_4^+, and their effects on pH_i, have been mathematically modeled (Boron & DeWeer, 1976*a*).

III. Active Transport Processes

A. Secondary Active Transport

The active transport of H^+ or of an ionic weak acid or base is any process which accomplishes net transport of a particular species against an electrochemical gradient. Active transport may be either "primary" (i.e., directly coupled to the hydrolysis of ATP or to electron transport) or "secondary" (i.e., energized by another solute moving down its electrochemical gradient). The reader is referred to the review by Aronson (1981) for a more general discussion of secondary active transport. The emphasis in this section will be on two transport processes, occurring in different cell types, which are known to regulate pH_i. In addition, brief mention will be made of Cl-HCO_3 exchange. The first two transport processes are responsible, in their respective cell types, for counteracting the acidifying tendencies of cellular metabolism and passive ion fluxes. Both processes are dependent on Na^+: the first is believed to exchange external Na^+ and HCO_3^- for internal Cl^- (and possibly H^+), and the second, to exchange external Na^+ for internal H^+. In both cases it can be shown that the energy derived from the influx of Na^+ down its steep electrochemical gradient is more than sufficient to drive the movement of the other ion(s). This hypothesis, that net transport should depend solely on the sum of the free energy gradients for each of the transported ions (ΔG_{net}), will be referred to here as the "gradient model". This hypothesis predicts that net transport should halt when $\Delta G_{net} = 0$, and should be reversed when ΔG_{net} is reversed. The data on the Na/HCO_3-Cl/H system suggest that reversal may be possible. However, the gradient model, and therefore of the designation "secondary active transport," is by no means firmly established.

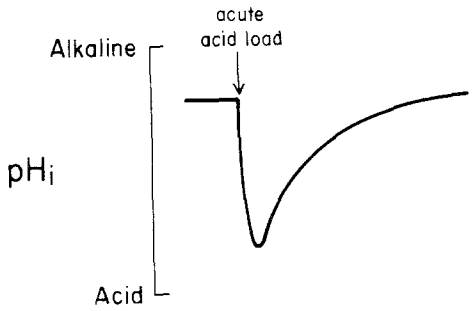

Fig. 4. Model experiment depicting the response of pH_i to an acute intracellular acid load, such as the injection of HCl or the application of CO_2. The exponential recovery is due to an active transport process

1. Na/HCO_3 – Cl/H Exchange

A transport system which apparently exchanges external Na^+ and HCO_3^- for internal Cl^- (and possibly H^+) is operative in squid giant axons, snail neurons, barnacle muscle, and possibly crayfish neurons (Moody, 1981). Although early data suggested that the transporter is very similar in the first three preparations, more recent evidence indicates that there are at least subtle differences among them; these will be pointed out below as this system is described in more detail.

The fundamental observation (*see* Fig. 4) is that when these cells are acutely acid-loaded by any of a number of means, the initial fall in pH_i is followed by a recovery toward its normal value of 7.3–7.4 (Thomas, 1974; Boron & DeWeer, 1976*a, b*; Boron, 1977). Such a recovery of pH_i from an acid load is due to an active transport process, which has been given the generic description "acid extrusion". It was soon discerned that the pH_i recovery in these cells requires HCO_3^- (Boron & DeWeer, 1976*b*; Thomas, 1976*a*; Boron, 1977), internal Cl^- (Russell & Boron, 1976; Thomas, 1977), and external Na^+ (Thomas, 1977; Russell & Boron, 1979; Boron, McCormick & Roos, 1981); and also that it can be blocked almost completely by the stilbene derivative SITS (Russell & Boron, 1976; Thomas, 1976*b*; Boron, 1977). These observations are consistent with each of the four models of Fig. 5. The top model has external Na^+ and HCO_3^- exchanging for H^+ and Cl^- (Thomas, 1977). In the second model, the exit of

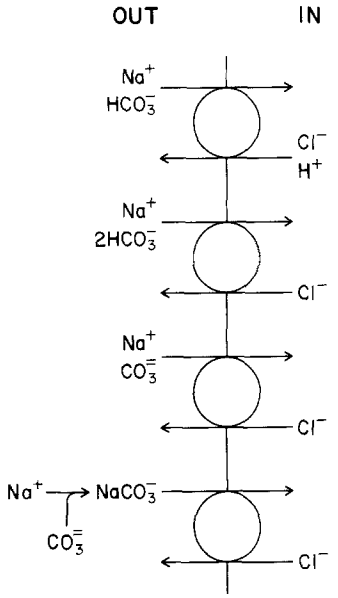

Fig. 5. Four models of acid extrusion in squid axons, snail neurons and barnacle muscle. All models predict electroneutrality and an absolute dependence on external Na^+, external HCO_3^- and internal Cl^-

H^+ is replaced by the entry of a second HCO_3^-. In the third model, a single CO_3^{2-} enters with Na^+, and in the fourth model (Becker & Duhm, 1978), the Na^+ and CO_3^{2-} form a complex prior to transport as the anion pair $NaCO_3^-$. All three models predict electroneutrality, consistent with data on membrane potential from both the snail neuron (Thomas, 1976*b*) and barnacle muscle (Boron, 1977). In addition, the schemes are equivalent thermodynamically. Thus, the models cannot be distinguished by their predicted effects on pH_i, or on Cl^- and Na^+ transport. Using isotopes, it has been shown that the recovery of pH_i from an acid load in squid axons is accompanied by a net efflux of Cl^- and an influx of Na^+ (Russell & Boron, 1979, 1982). Furthermore, these Cl^- and Na^+ fluxes have the same properties as the recovery of pH_i from an acid load: they (i) are blocked by SITS (ii) are dependent on HCO_3^-, and (iii) occur only when pH_i is depressed below its normal value. Also, the net Cl^- efflux requires external Na^+ and the Na^+ influx requires internal Cl^-. Finally, the stoichiometry is as predicted by the models of Fig. 5: one equivalent of Na^+ taken up for each equivalent of Cl^- lost, and for each two equivalents of acid removed from or alkali added to the cell.

Several characteristics of this transport system are worth examining in greater detail: its dependence on (a) the concentrations of various substrates, (b) pH_o, (c) pH_i, and (d) ATP, as well as (e) its sensitivity to various inhibitors.

(a) Dependence on Substrates. As demonstrated in squid axons (Russell & Boron, 1976) and in snail neurons (Thomas, 1977), this transporter absolutely requires intracellular Cl^-, suggesting that acid extrusion is tightly coupled to Cl^- efflux. In squid axons[1] (Russell & Boron, 1982), acid extrusion is accompanied by a net Cl^- efflux, obtained from the difference between isotopic Cl^- influx and efflux, of 3.8 pmol·cm^{-2}·sec^{-1}. This isotopic approach also has demonstrated a net Cl^- efflux accompanying acid extrusion in barnacle muscle (Boron et al., 1978), whereas Thomas (1977) has used Cl-sensitive microelectrodes to confirm that a fall of intracellular Cl^- activity accompanies acid extrusion in snail neurons. These results indicate that internal Cl^- is truly a substrate of the transport system. The dependence of the acid extrusion rate on $[Cl^-]_i$ follows Michaelis-Menten kinetics in squid axons[2] (Russell & Boron, 1982), with an apparent Michaelis constant (K'_m) of 83 ± 15 mM. It is noteworthy that this K'_m value is in the normal $[Cl^-]_i$ range for squid axons, suggesting that changes in $[Cl^-]_i$ could have important consequences for pH_i regulation.

Acid extrusion also has an absolute requirement for external Na^+, as demonstrated in experiments on snail neurons (Thomas, 1977), barnacle muscle (Boron et al., 1981) and squid axons (Russell & Boron, 1979, 1982). The first indication that Na^+ efflux accompanies acid extrusion was Thomas' (1977) observation, made with Na-sensitive microelectrodes, that the intracellular Na^+ activity transiently rises during periods of acid extrusion. Subsequent experiments on squid axons[1] (Russell & Boron, 1982), in which the net Na^+ flux was calculated from radioisotopic Na^+ fluxes, showed that acid extrusion is accompanied by a net Na^+ influx of 3.4 pmol·cm^{-2}·sec^{-1}. This is very similar to the value for the aforementioned net Cl^- efflux associated with acid extrusion. Furthermore, this isotopically measured Na^+ flux was found to have all the properties expected of the pH_i-regulating system: dependence on (i) external HCO_3^-, (ii) internal Cl^-, (iii) internal ATP, and (iv) a low pH_i, as well as (v) sensitivity to SITS. Thus external Na^+ is also a substrate of this transport system. The K'_m for external Na^+ is 59 ± 6 and 77 ± 13 mM, respectively, in barnacle muscle[2] (Boron et al., 1981) and squid axons[2] (Russell & Boron, 1982).

[1] Condition: $[Na^+]_o = 425$ mM, $[HCO_3^-]_o = 12$ mM, $pH_o = 8.0$, $[Cl^-]_i = 150$ mM, $pH_i = 6.5$, and $T = 16°$ C.

[2] Conditions: $[Na^+]_o = 425$ to 440 mM, $[HCO_3^-]_o = 10$ to 12 mM, $pH_o = 8.0$, $pH_i = 6.7$, and $T = 22°$ C.

Finally, this pH_i-regulating system requires HCO_3^- or a related ion. Evidence for the involvement of HCO_3^- was provided in experiments on squid axons (Boron & DeWeer, 1976*b*) and snail neurons (Thomas, 1976*a*), in which it was shown that the rate of recovery of pH_i from an acute intracellular acid load is much higher in the presence of HCO_3^- and CO_2 than in their absence. Unfortunately, the evidence for transport of HCO_3^- can be no more direct than demonstrating HCO_3^- dependence of the pH_i recovery; HCO_3^- fluxes cannot be studied with isotopes because the isotopic label would quickly exchange with CO_2. The K'_m for external HCO_3^- is 4.2 ± 1.1 and 2.3 ± 0.2 mM, respectively, in barnacle muscle[2] (Boron et al., 1981) and squid axons[2] (Russell & Boron, 1982). Note that for both external Na^+ and HCO_3^-, the K'_m values are substantially lower than the normally prevailing substrate concentrations.

The rate of pH_i recovery from an acute intracellular acid load can be used to calculate the "net equivalent HCO_3^- flux", which includes the net influx of HCO_3^- and/or CO_3^{2-}, as well as the efflux of H^+. In a preparation like the snail neuron or barnacle muscle, for which the true membrane surface area is unknown, the net equivalent HCO_3^- flux can be obtained in the units mol·liter^{-1}·sec^{-1} merely by multiplying the rate of pH_i recovery (pH units·sec^{-1}) by the empirically determined intracellular buffering power (mol strong base·liter^{-1}·pH unit^{-1}). In a preparation like the squid axon, in which the membrane surface area can be accurately measured, HCO_3^- transport can be expressed as a flux per area: mol·m^{-2}·sec^{-1}. The net equivalent HCO_3^- flux in squid axons (Russell & Boron, 1982) is 7.5 ± 0.6 pmol·cm^{-2}·sec^{-1}. This value should be compared to the aforementioned values for the net Cl^- efflux (i.e., 3.8 pmol·cm^{-2}·sec^{-1}) and net Na^+ influx (i.e., 3.4 pmol·cm^{-2}·sec^{-1}) mediated by the pH_i-regulating system. These data are thus consistent with the 1:1:2 stoichiometry predicted by each of the models of Fig. 5.

(b) Dependence on pH_o. The acid extrusion rate in squid axons decreases when pH_o and $[HCO_3^-]_o$ are simultaneously lowered at constant pCO_2 (Boron & DeWeer, 1976*b*). This is not unexpected, of course, given the aforementioned dependence of the transport system on $[HCO_3^-]_o$ alone. The question of whether pH_o per se affects acid extrusion can be approached by measuring the inhibition of acid extrusion as pH_o is lowered either by lowering $[HCO_3^-]_o$ at constant pCO_2 or by raising pCO_2 at constant $[HCO_3^-]_o$. The results of such an experiment on barnacle muscle (Boron, McCormick & Roos, 1979) show that inhibition of acid extrusion is threefold greater when $[HCO_3^-]_o$ and pH_o are decreased concurrently, as opposed to when pH_o is decreased alone. Acid extrusion is thus influenced by changes in pH_o, independently of changes in $[HCO_3^-]_o$.

(c) Dependence on pH_i. Perhaps the most striking characteristic of the pH_i-regulating system is its responsiveness to intracellular acid loads, i.e., decreases in pH_i. The $Na/HCO_3 - Cl/H$ system is relatively inactive at normal pH_i values, but is increasingly stimulated as pH_i is reduced (Boron et al., 1979). The relative inactivity at normal pH_i values is illustrated by an experiment in which a fresh barnacle muscle fiber is exposed to SITS: pH_i is stable even though the drug completely blocks the pH_i-regulating system (Boron et al., 1979). If SITS does not otherwise affect pH_i, this observation implies that (i) the baseline rate of intracellular acid loading is very low and (ii) the baseline rate of acid extrusion must likewise be very low. The pH_i-regulating mechanism behaves quite differently, however, when pH_i is reduced. In experiments in which he iontophoretically injected HCl into snail neurons, Thomas (1976*b*) noted that the abrupt decline in pH_i is followed by its recovery to its initial value (similar to the model experiment in Fig. 4). This recovery occurs along an exponential time course, its rate slowing to zero as pH_i approaches a normal level. The exponentiality implies that at any point during the recovery, the rate of pH_i recovery, dpH_i/dt, is given by:

$$dpH_i/dt = k(pH'_i - pH_i), \tag{1}$$

where k is the rate constant and pH'_i is the asymptotic pH_i (i.e., the baseline or normal pH_i value). Since the acid extrusion rate, J (given in mol·liter^{-1}·sec^{-1}), is merely the product of the pH_i recovery rate and the intracellular buffer power, β, it follows that:

$$J = k\beta\,(pH'_i - pH_i), \tag{2}$$

provided that β is independent of pH_i over the pH_i range in question. In other words, as pH_i is reduced below pH'_i, J increases proportionately. Such a linear dependence of J on pH_i has been demonstrated in barnacle muscle[3] (Boron et al., 1979):

$$J = C\,(pH'_i - pH_i). \tag{3}$$

[3] In these cells, β is not invarient of pH_i, but neither is the recovery of pH_i from an acid load perfectly exponential. Therefore, Eq. (2) is not valid. Nevertheless, the two effects cancel each other to provide a linear dependence of J on pH_i.

Additional evidence for the pH_i dependence of the pH_i-regulating system is provided by experiments in which Cl^- fluxes were measured with ^{36}Cl. In addition to net acid extrusion, the barnacle muscle's pH_i-regulating system also mediates Cl–Cl exchange. As pH_i is reduced, both acid extrusion and Cl–Cl exchange are increased (Boron et al., 1978).

Equation 3 presumably also describes the dependence of acid extrusion rate on pH_i when pH_o or the concentration of one of the substrates is altered. This was confirmed in experiments on barnacle muscle (Boron et al., 1979) in which the recovery of pH_i from an acid load was monitored under different conditions of $[HCO_3^-]_o$ and pH_o (constant pCO_2): 40 mM HCO_3^-/pH 8.6, 10 mM HCO_3^-/pH 8.0, and 2.5 mM HCO_3^-/pH 7.4. As expected, J fell as $[HCO_3^-]_o$ and pH_o were reduced. However, this effect was accounted for solely by differences in the proportionality factor C; pH_i' was not appreciably affected, ranging between 7.3 and 7.4. This suggests that at pH_i values above pH_i', transport ceases; as pH_i falls below this threshold, transport is gradually stimulated.

The threshold pH_i does not merely reflect that pH_i at which the transport system's requirements exceed the available energy: at pH_o 8.0, for example, pH_i recovery halts at less than 7.4, even though there is sufficient energy in the ion gradients to drive pH_i well above 8.0. It is also clear that the transport system is not maintaining a particular $[HCO_3^-]_i/[HCO_3^-]_o$ ratio or a $(pH_o - pH_i)$ difference: pH_i recovery halts (i.e., the acid extrusion rate fell to zero) at pH 7.3 to 7.4 over a wide range of values of $[HCO_3^-]_o$ and pH_o. The simplest explanation for the data is that the transport process is gated by pH_i in a manner analogous to that in which certain ionic channels are gated by voltage. Net transport requires (i) sufficient energy in the ion gradients to drive the system, and (ii) a pH_i low enough to open the gate. The mechanism of the gating could be an inward-facing allosteric site which activates the transporter when $[H^+]$ increases. A simple Michaelis-Menten dependence on $[H^+]_i$ is less likely, given the lack of a sigmoidal dependency of acid extrusion rate on pH_i.

Note that the pH_i value at which pH_i levels off following recovery from an acid load is not necessarily the same as the threshold of the transport system. The barnacle muscle is a very special case inasmuch as the underlying rate of intracellular acid loading is very low. Thus, when pH_i levels off following recovery from an acid load, J is very nearly zero. This can be demonstrated by exposing a muscle fiber to SITS after the pH_i recovery is complete, a treatment which causes pH_i to drift only very slowly in the acid direction. In rapidly metabolizing cells, or in cells with a high passive H^+ influx or HCO_3^- efflux, the underlying rate of acid loading may be much larger. Hence, the rate of acid extrusion needed to maintain a stable pH_i would also be much higher. When pH_i would recover from an acid load in this case, pH_i would level off far below the true threshold for the transport system. The discrepancy between the true threshold and the apparent threshold reflects the degree of stimulation of the pH_i-regulating system required to balance the underlying acid-loading rate.

(d) Dependence on ATP. In the squid axon, the pH_i-regulating system is blocked by the metabolic inhibitors cyanide and 2,4-dinitrophenol (Boron & DeWeer, 1976*b*). Returning ATP to the system, even in the continued presence of cyanide, allows the system to resume transport (Russell & Boron, 1976, 1982). The role of ATP is unclear, since there is sufficient energy in the Na^+ gradient to account for the observed HCO_3^- and Cl^- transport. Perhaps ATP is permissive, being required for an essential phosphorylation reaction. The only other preparation in which ATP dependence has been examined is the snail neuron. Thomas (1976*b*) found that when the cells were poisoned with the mitochondrial uncoupler to an extent sufficient to block the Na^+ pump, there was no effect on the neuron's pH_i-regulating system. Unless this pH system has a very high affinity for ATP, Thomas' results would argue against any involvement of ATP in the snail neuron's pH_i-regulating system. In addition, Thomas (1982) has found that injection of orthovanadate is without effect. ATP dependence thus appears to be one area where the various Na/HCO_3–Cl/H transport systems differ.

The ATP dependence of the squid axon's pH_i-regulating mechanism may provide a mechanism for turning off energy-dissipating reactions at times when cellular ATP levels are falling. Since acid extrusion in the squid axon involves the uptake of Na^+, acid extrusion indirectly leads to an increased Na–K pump rate and, therefore, to an increased rate of ATP consumption. If Na-coupled systems were active only at a relatively high [ATP], then a fall in $[ATP]_i$, such as might occur in severely energy-starved cells, would lead to a halt in transport processes which would otherwise dissipate the Na^+ gradient. It is of interest to note that two other transport systems of the squid axon are known to be both external Na^+

dependent and ATP dependent: NaCl cotransport (Russell, 1979) and Na−Mg exchange (DeWeer, 1976). In the latter case, internal ATP increases the carrier's apparent affinity for Mg^{++} (Mullins et al., 1977).

(e) Sensitivity to Inhibitors. The pH_i-regulating systems of squid, snail and barnacle are characterized by their sensitivity to the stilbene derivatives, such as SITS. The stilbenes had previously been known to block the erythrocyte anion-exchange system (*see* Cabantchik, Knauf & Rothstein, 1978 for a review) when Thomas (1976*b*) showed that application of either SITS or 4,4′-diisothiocyanostilbene-2,2′-dilsulfonate (DIDS) blocked the ability of the snail neuron's pH_i to recover from an acute acid load. The ability of SITS to block acid extrusion was confirmed in both squid axons (Russell & Boron, 1976) and barnacle muscle (Boron, 1977). Apparently, both SITS and DIDS react covalently (at the isothiocyano moiety) with a free amino group on the transport mechanism. However, such a covalent reaction is clearly not necessary, since the congener 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), which cannot react covalently, reversibly inhibits acid extrusion in squid axons (Russell & Boron, 1979). Thus, as is apparently the case for the erythrocyte anion exchanger, an electrostatic interaction between a positively charged area on the transporter and the sulfonate groups on the stilbene nucleus is sufficient to halt transport. The covalent reaction between the isothiocyano moiety(ies) of SITS and DIDS occurs more slowly and makes the inhibition permanent. Interestingly, two other amino-reactive agents, pyridoxal phosphate and *p*-isothiocyanatobenzenesulfonate, also inhibit the pH_i-regulating mechanism in barnacle muscle (Boron, 1977). Acid extrusion is also reversibly inhibited by the "loop diuretic" furosemide in both barnacle muscle (Boron et al., 1978) and squid axons (Russell & Boron, 1979). In the latter case, the inhibition amounts to ~85% at a drug concentration of 0.6 mM. Furosemide is much less specific than the stilbenes. For example, in squid axons this compound is a potent inhibitor of the NaCl cotransport system (J.M. Russell, *personal communication*), which is unrelated to the pH_i regulator.

Although there are many similarities among the pH_i-regulating mechanism of squid, snail and barnacle, apparent differences also exist. The difference in ATP dependence between squid axons and snail neurons has already been noted. Another difference is the cation specificity of the extracellular cation (i.e., Na^+) site. In barnacle muscle, Li^+ supports acid extrusion about two-thirds as well as Na^+ (Boron et al., 1981), whereas in squid axons, Li^+ does not support acid extrusion at all (Russell & Boron, 1982). A third area of dissimilarity is the ability of the pH_i-regulating system to mediate the self-exchange of Cl^-. The barnacle muscle's pH_i regulator exchanges Cl^- for Cl^- at a rate which increases as pH_i is reduced (Boron et al., 1978), whereas the squid axon's does not appear to mediate Cl−Cl exchange, even under conditions of extreme acid loading (Russell & Boron, 1982). Lastly, both acid extrusion and Cl−Cl exchange are stimulated by cyclic AMP in barnacle muscle (Boron et al., 1978), while in squid axon this nucleotide is without appreciable effect (Russell & Boron, *unpublished*).

A final area of interest concerning the pH_i-regulator of squid, snail and barnacle is its apparent ability to be reversed (a net efflux of HCO_3^- and Na^+, a net influx of Cl^- and possibly H^+). In barnacle, sharply reducing pH_o (under conditions of intracellular acid loading) causes a fall in pH_i (Boron, 1977) which is dependent on HCO_3^- and blocked by SITS (Boron et al., 1979). Such an intracellular acidification is accompanied by a rise of intracellular Cl^- activity in the snail neuron, consistent with the reversed operation of the pH_i regulator (Thomas, 1980). Recent work on barnacle muscle (Russell, Brodwick & Boron, *unpublished*) has shown that reversal of the pH_i regulator (i) can be initiated by removal of external Na^+ (at high internal Na^+) or by greatly lowering pH_o, (ii) is dependent on external Cl^-, and (iii) is directly related to $[Na^+]_i$.

2. Na−H Exchange

Na−H exchangers have been identified, or at least tentatively identified, in a number of intact cells, including mouse soleus muscle (Aickin & Thomas, 1977b), sheep cardiac Purkinje fibers (Dietmar & Ellis, 1980), frog muscle (Abercrombie & Roos, 1981; Moore, 1981), crayfish neurons (Moody, 1981), salamander proximal tubule cells (Boron & Boulpaep, 1982), *Amphiuma* erythrocytes (Cala, 1980; Siebens & Kregenow, 1980); and the MDCK cell line (Rindler, Taub & Saier, 1979). In addition, this transport system has been studied in membrane vesicles isolated from intestinal (Mürer, Hopfer & Kinne, 1976) and renal (Mürer et al., 1976; Kinsella & Aronson, 1980) brush border membranes. Studies with pH-sensitive microelectrodes have shown that the Na−H exchanger of the aforementioned muscles, Purkinje fibers and proximal tubule cells clearly exhibits the one prop-

erty crucial for a pH_i-regulator: sensitivity to intracellular acid loading. After an acute acid load, pH_i recovers toward its normal value following a time course which is approximately exponential. This exponentiality implies that, as is the case for the previously discussed $Na/HCO_3-Cl/H$ system, the rate of $Na-H$ exchange increases linearly as pH_i falls.

The initial evidence for a discreet $Na-H$ exchanger was provided by Mürer et al. (1976) in their experiments on membrane vesicles isolated from the brush borders of rat kidney and intestine. They found that Na^+ uptake by the vesicles is stimulated by lowering pH_i, and accompanied by a fall of pH_o. Later work with ion-sensitive electrodes by Aickin and Thomas (1977*b*) on mouse soleus muscle showed that the recovery of pH_i from acute intracellular acid loads depends on external Na^+ and is accompanied by a rise of intracellular Na^+ activity. Furthermore, the pH_i recovery is inhibited by the diuretic amiloride. Similar observations have been made in sheep cardiac Purkinje fibers (Deitmer & Ellis, 1980), and salamander proximal tubule cells (Boron & Boulpaep, 1982). This presumed $Na-H$ exchanger appears to be unrelated to the $Na/HCO_3-Cl/H$ system. In the proximal tubule cells, for example, the pH_i recovery from an acid load (i) occurs in the nominal absence of HCO_3^-, (ii) is unaffected by stilbene derivatives, (iii) is not influenced by removal of Cl^-, and (iv) in the presence of Cl^- is not accompanied by changes in intracellular Cl^- activity. Pharmacologically, the $Na-H$ exchanger is distinguished from coupled $Na/HCO_3-Cl/H$ transport by the former's sensitivity to amiloride and insensitivity to stilbenes. Although Aickin & Thomas (1977*b*) reported that 0.1 mM amiloride blocked $Na-H$ exchange nearly completely in mouse muscle, data on other preparations indicate that amiloride is generally not so potent. Kinsella and Aronson (1980), working with membrane vesicles isolated from the brush borders of rabbit kidneys, found that amiloride is apparently competitive with Na^+; the apparent k_m for Na is ~5 mM, the apparent k_I for amiloride, ~16 μM. These data predict that at 140 mM Na^+, 0.1 mM amiloride should inhibit $Na-H$ exchange by only ~20%. Indeed, 2 mM amiloride is required to produce ~75% inhibition of $Na-H$ exchange in salamander proximal tubule cells when $[Na^+]_o$ is 100 mM, though only 1 mM amiloride is nearly totally effective at 20 mM Na^+. In Purkinje fibers (Deitmer & Ellis, 1980), 1 mM amiloride inhibits 67–80% of $Na-H$ exchange at normal external Na^+ levels. Rindler et al. (1979), studying ^{22}Na uptake in MDCK cells, found that the apparent K_I for amiloride is virtually the same as the Kinsella-Aronson value for vesicles.

The ^{22}Na studies of Rindler et al. (1979) and of Kinsella and Aronson (1980) indicate that the same transporter which mediates $Na-H$ exchange may also mediate $Na-Na$ exchange. The former authors found that under steady-state conditions, a substantial fraction of ^{22}Na uptake depends on internal Na^+, whereas the latter authors demonstrated that a large component of ^{22}Na requires external Na^+, though the amiloride sensitivity of these fluxes has yet to be examined. These observations raise two related questions: (i) What is the dependence of $Na-Na$ exchange on pH_i? (ii) Does $Na-Na$ exchange occur at the expense of $Na-H$ exchange? These studies with ^{22}Na have also helped define the cation specificity of this carrier. Both Rindler et al. and Kinsella and Aronson found that ^{22}Na uptake is competitively inhibited by external Li^+; the latter group showed that K^+, Rb^+, Cs^+ and choline are without effect.

Three novel aspects of $Na-H$ exchangers are also worthy of consideration: activation of $Na-H$ exchange in frog muscle, volume regulation in *Amphiuma* erythrocytes, and the mode of acid secretion in epithelia.

(*a*) Activation of $Na-H$ exchange in frog muscle. In 1977, Bolton and Vaughan-Jones found that when frog muscle fibers are exposed to 5% CO_2/15 mM HCO_3^- (pH_o 7.2), pH_i fails to recover from the resultant intracellular acidification. This failure of pH_i to recover could have been due to a passive efflux of HCO_3^- which matched the rate of acid extrusion. Alternatively, the HCO_3^- efflux could have been low, and the pH_i-regulating mechanisms may simply have been inactive under the conditions of the experiment. Recently, Abercrombie and Roos (1981, 1982), in experiments in which they monitored pH_i in frog semitendinosous muscles with pH-sensitive microelectrodes, confirmed that pH_i fails to recover from a CO_2-induced acid load. They found, however, that when the cells are depolarized, pH_i readily recovers. The requisite depolarization can be accomplished in any of four ways: (i) by 50 mM $[K^+]_o$ at normal $[Cl^-]_o$; (ii) by 50 mM $[K^+]_o$ at a normal $[K^+]_o \cdot [Cl^-]_o$; (iii) by 0.5 mM $[Ba^{++}]_o$ at 2.5 mM $[K^+]_o$ and 5.9 mM $[Cl^-]_o$; and (iv) by normal Ringer's after 1 hr exposure to 50 mM $[K^+]_o$ at normal $[Cl^-]_o$.

This depolarization-activated pH_i recovery is nearly abolished by removal of external Na^+. In cells depolarized by method (i), which raises $[Cl^-]_i$, 0.5 mM SITS slows recovery by 1/3 to 1/2, and

1 to 2 mM amiloride slows recovery by about 1/3. When, on the other hand, depolarization is achieved by method (ii) (i.e., without elevating $[Cl^-]_i$), the pH_i recovery is insensitive to SITS but is still inhibited by amiloride. These results suggest that the frog muscle fiber has two separate mechanisms for extruding acid: an amiloride-sensitive Na−H exchanger, and a SITS-sensitive Cl−HCO_3 (or OH^-) exchanger which is active only when $[Cl^-]_i$ is elevated. The authors found a somewhat different response in fibers depolarized in hypertonic Ringer's solutions (250 mM mannitol added). In this case, pH_i recovery is sensitive only to amiloride, even when $[Cl^-]_i$ is elevated by depolarizing the fibers in normal Cl^-. It is uncertain whether this pH_i regulation under hypertonic conditions is related to that seen in the *Amphiuma* red cells as a result of volume changes (*see* next section).

Another observation concerning pH_i regulation in frog muscle has been made by Moore (1979, 1981). When insulin is applied to frog sartorius muscle, pH_i (as measured with the weak acid DMO) rises by ~0.1 over the course of ~2 hr. This intracellular alkalinization is accompanied by an increase in $[Na^+]_i$, provided ouabain is present to block the Na−K pump. Inasmuch as the rise in pH_i requires Na^+, and the increase in both pH_i and $[Na^+]_i$ are inhibited by amiloride, the insulin-activated transport system may be a Na−H exchanger.

***(b)* Na—H exchange and cell-volume regulation.** A second novel aspect of Na−H exchange is the possible involvement of this transport mechanism in cellular volume regulation. When *Amphiuma* erythrocytes are placed in a hypertonic medium, their initial shrinkage is followed by a gradual re-expansion over a one- to three-hour period. Siebens & Kregenow (1978, 1980) have shown that this re-expansion is accompanied by a more than 30-fold increase of Na^+ influx, which is independent of both K^+ and Ca^{++}, and reversibly inhibited by amiloride (apparent $K_I = 2 \times 10^{-6}$ M). Furthermore, it is accompanied by a rise in pH_i as measured in frozen-thawed cells (Siebens & Kregenow, 1980), and a fall in pH_o (Cala, 1980). These data all support the existence of a Na−H exchanger. Cala (1980) has shown that the re-expansion of the cells is brought about by the net uptake of Na^+, Cl^- and osmotically obligated H_2O. He proposes that a Na−H exchanger mediates the Na^+ uptake, while a Cl−HCO_3 exchanger mediates the net Cl^- uptake. Measurements of V_m indicate that the processes are electroneutral (Cala, 1980). When Cl−HCO_3 exchange is blocked by DIDS, the cells still re-expand, but by Na−H exchange alone. Measurements made with DIDS present suggest that the Na/H stoichiometry is 1:1. Should, as the data suggest, the volume expansion of these cells be mediated by a Na−H exchanger, two crucial questions arise: (i) Is this the same Na−H exchanger responsible for pH_i regulation in other cells? And (ii) if so, how are pH_i and volume regulation interrelated? One possibility is that volume changes induce shifts in pH_i. If cell shrinkage were to cause a fall in pH_i, for example, one would expect the pH_i-regulating mechanism to respond by accelerating Na−H exchange. Indeed, Ellis and Thomas (1976) have reported that exposing sheep cardiac Purkinje fibers to a hyperosmotic Ringer's (containing an additional 100 mM sucrose) causes a rapid and reversible fall of pH_i (~0.1). It would thus seem that since pH_i is a potential indicator of cell volume, as suggested by Cala (1980), a Na−H exchange could regulate volume in virtue of regulating pH_i. If Na−H were indeed to serve two functions, pH_i regulation and volume regulation, the question which arises is whether the cell can regulate pH_i without altering cell volume. If all decreases in pH_i are accompanied by cell shrinkage and vice versa, no problem would arise, since Na−H exchange would simultaneously and necessarily tend to restore both pH_i and cell volume to normal. If, on the other hand, the cell could be acid loaded without sustaining a fall in cell volume, then restoration of a normal pH_i would tend to raise cell volume above normal. The conflicting demands of cell-pH and cell-volume regulation could be reconciled only if a second cell-volume regulator, one responsive to increases in cell volume, acted to maintain a stable pH_i even in the face of Na−H exchange. Such a mechanism is known to exist in *Amphiuma* erythrocytes (Cala, 1980).

***(c)* Mode of acid secretion in epithelia.** A third novel aspect of Na−H exchange is the role it plays in net acid secretion in certain epithelia. As noted above, Na−H exchangers have been identified at the luminal surface of intact cells of the salamander proximal tubule (Boron & Boulpaep, 1982). These cells also have a Na−H exchanger at the basolateral membrane, but this can be neglected for the purposes of this discussion. Work with ion-sensitive microelectrodes (Boron & Boulpaep, 1982) indicates that the Na–H exchanger functions as a classical pH_i regulator: the Na−H exchange rate increases in response to intracellular acid loads, and slows as pH_i increases. When proximal tubules

are incubated in CO_2/HCO_3^--free Ringer's, pH_i is relatively high (probably approaching the pH_i threshold for the Na−H exchangers), and the Na−H exchange rate is probably rather low. When the cells are exposed to a CO_2/HCO_3^--containing Ringer's at constant external pH, pH_i rapidly falls by 0.15 to 0.20, but fails to recover. The initial fall in pH_i, due to the influx of CO_2 and the subsequent formation of H^+ and HCO_3^- intracellularly, represents an acute intracellular acid load, which in turn should stimulate the cell's pH_i-regulating system (i.e., Na−H exchange). The failure of pH_i to recover indicates that HCO_3^- (or a related species) leaks out of the cell across the basolateral (or blood-side) membrane as rapidly as the pH_i-regulating mechanism can exchange Na^+ for H^+. The H^+ extruded from the cell across the luminal membrane constitutes transcellular acid secretion. Thus, acid secretion is merely a by-product of pH_i regulation: the efflux of HCO_3^- across the basolateral membrane provides a sustained intracellular acid load to which the Na−H exchanger responds by extruding H^+ across the luminal membrane. This model, which treats epithelial acid secretion as a special case of pH_i regulation, can be extended to any epithelial cell which transports acid or base. For the proximal tubule, the model predicts that any treatment which enhances basolateral HCO_3^- efflux should lower pH_i and consequently stimulate luminal Na−H exchange (i.e., acid secretion). Conversely, maneuvers which reduce basolateral HCO_3^- efflux should reduce the intracellular acid load and allow Na−H exchange to raise pH_i, thereby reducing the steady-state Na−H exchange rate. Indeed, Kleinman et al. (1980) have shown that blockade of basolateral HCO_3^- transport by SITS causes an increase of pH_i. Although this SITS-induced rise in pH_i reflects an enhancement of the cell's ability to regulate its pH to a relatively alkaline value, the situation is obviously counterproductive for transepithelial acid secretion.

3. Cl−HCO_3 Exchange

The most extensively studied Cl−HCO_3 exchanger, by far, is that of the erythrocyte. For recent reviews, consult Cabantchik et al. (1978) and Knauf (1979). Information on Cl−HCO_3 exchange in other cell types is much less detailed. For mouse soleus muscle (Aickin & Thomas, 1977*b*) and sheep cardiac Purkinje fibers (Vaughan-Jones, 1979; Dietmer & Ellis, 1980), data obtained with ion-sensitive microelectrodes suggest that a Cl−HCO_3 exchanger exists in parallel with the Na−H exchanger discussed in the preceding section. There are three lines of evidence for HCO_3^- transport in mouse soleus muscle: (i) the rate of pH_i recovery from an acid load is reduced about 30% by removal of HCO_3^- or by addition of SITS, (ii) the effect of SITS is additive to that of either Na^+ removal or amiloride application, and (iii) application of amiloride increases the Q_{10} of the residual pH_i recovery to 6.9 from a pre-amiloride value of only 2.6. This last observation suggests that amiloride nullifies a normally dominant, low-Q_{10} Na−H exchanger, thereby leaving intact a low-capacity, high-Q_{10} HCO_3^- transporter. Although there is no data indicating that Cl^- is involved in this HCO_3^- system in mouse muscle, there is excellent evidence that this is the case in the Purkinje fibers (Vaughan-Jones, 1979). In this preparation, removal of external Cl^- produces a reversible decrease in intracellular Cl^- activity, which is accompanied by a rise in pH_i. Calculations show that the movement of Cl^- is accompanied by the movement of HCO_3^- in the opposite direction, with a 1:1 stoichiometry. SITS blocks both the changes in pH_i and intracellular Cl^- activity. These data point to the existence of a reversible HCO_3−Cl exchanger. Inasmuch as an analysis of the energy requirements of this transport system have yet to be undertaken, it is unclear whether the observed Cl−HCO_3 exchange can be accounted for by an electroneutral exchanger fueled only by Cl^- and HCO_3^- gradients. As to the physiological role of a HCO_3−Cl exchanger in cells which already possess a Na−H exchanger, it seems likely that the primary function of the HCO_3−Cl system is to regulate $[Cl^-]_i$. Since $[Cl^-]_o/[Cl^-]_i$ is generally greater than $[HCO_3^-]_o/[HCO_3^-]_i$, a passive and neutral Cl−HCO_3 exchanger would normally mediate Cl^- uptake and HCO_3^- extrusion. Although this is beneficial for cells requiring the intracellular Cl^- activity to be above the equilibrium value, such an exchange also imposes an intracellular load upon the cell, because of the HCO_3^- efflux. This loss of HCO_3^- would presumably be compensated for by the activity of the cell's primary pH_i regulator: the Na−H exchanger. Thus, with regard to pH_i, the Cl−HCO_3 and Na−H exchangers would be antagonistic under normal circumstances. However, in certain cases, a Cl−HCO_3 exchanger could act as an adjunct pH_i regulator. For example, if pH_i were raised above normal, Cl−HCO_3 exchange would presumably accelerate and thereby help return pH_i toward normal. This has actually been observed in the Purkinje fibers (Vaughan-Jones, 1982). Such pH_i regulation on the alkaline side of the normal pH_i is of interest because the classic pH_i regulators (i.e.,

coupled $Na/HCO_3-Cl/H$ exchange and $Na-H$ exchange) regulate pH_i only on the acid side. An electroneutral $Cl-HCO_3$ exchanger could also participate in acid extrusion, but only if pH_i falls sufficiently to raise $[HCO_3^-]_o/[HCO_3^-]_i$ above $[Cl^-]_o/[Cl^-]_i$. Such was apparently the case in the mouse soleus muscle study of Aickin and Thomas (1977*b*).

B. Primary Active Transport

The movement of H^+ is described as primary active transport if that flux is directly coupled to ATP hydrolysis or to electron transport. The effector of H^+ primary active transport is a proton pump, of which two general types can be described: electroneutral and electrogenic. An example of an electroneutral H^+ pump is the gastric $K-H$ exchanger (Sachs et al., 1976). This transporter, located at the parietal cell's apical membrane, exchanges external K^+ for internal H^+, using energy derived from the hydrolysis of ATP. For excellent discussions of this transport system, consult the reviews by Sachs, Spenney and Lewin (1978) and Forte, Machen and Obrink (1980). Electrogenic proton pumps exist in the membranes of plant cells, bacteria, and fungi, as well as chloroplasts and mitochondria. In these preparations, the proton pump generates an electrochemical H^+ gradient which in turn is used to energize various secondary active transport processes. The reader is directed to a number of recent reviews of these subjects.

An electrogenic proton pump is also present in certain "tight" epithelia, such as turtle bladder and, presumably, the mammalian distal nephron. In both cases, it is thought that only a certain cell type within the epithelium, the mitochondria-rich cells in the turtle bladder and the morphologically similar intercalated cells in the distal nephron, actually possesses the H^+ pump. Even though these cells make up only a fraction of total cells within the epithelium (e.g., about 15% in the turtle bladder; *see* Husted et al., 1981), they are responsible for the generation of a sizeable transepithelial pH gradient. As is the case in the proximal tubule, the secretion of acid from the basolateral (or blood-side) to the luminal solution in these tight epithelia must be a two-step process: (i) acid enters the cell across the basolateral membrane, and (ii) acid exits the cell across the luminal membrane. It is this second step which is mediated by the H^+ pump of the mitochondria-rich cells. The properties of this epithelial H^+ pump are discussed in detail by Steinmetz and Andersen (1982) in a recent Topical Review. To summarize briefly, the pump utilizes the energy of ATP hydrolysis to eject protons across the luminal membrane against an electrochemical H^+ gradient of up to ~ 180 mV. The pump appears to be more akin to the fungal H^+ pump than the mitochondrial one. In particular, both the epithelial and fungal H^+ pumps are sensitive to vanadate and resistant to oligomycin, just opposite to the pattern obtaining for the mitochondrial H^+ pump.

With regard to pH_i regulation within "tight" epithelial cells, two questions arise. Firstly, what is the nature of H^+ transport in the cells that make up the bulk of the epithelium, the granular cells in the turtle bladder (Husted et al., 1981) and the morphologically similar principal cells in the mammalian distal nephron (Kaissling & Kriz, 1979)? Presumably these principal cells do not possess a H^+ pump and, therefore, could not engage in transepithelial acid secretion. However, they probably have the same requirement for pH_i regulation as do nonepithelial cells, such as nerve or muscle. We might therefore expect to find that pH_i is regulated by a secondary active transport system, such as a $Na-H$ exchanger or a $Na/HCO_3-Cl/H$ exchanger. Such a secondary active transport system would probably be located at the basolateral membrane, where Na^+ and H^+ gradients are favorable for extrusion of H^+ (or uptake of HCO_3^-). If such a transporter were also located as the luminal membrane, however, it would not be able to contribute to acid extrusion under conditions of low luminal pH, since there would be insufficient energy in the Na^+ gradient to drive, for example, $Na-H$ exchange. If such a luminal exchanger were easily reversible, then, under conditions of low luminal pH, it would mediate acid uptake by the cell. Since this acid would be extruded by the basolateral pH_i regulator, such a luminal H^+ "leak" would short-circuit a portion of the acid secreted by the mitochondria-rich or intercalated cells. Therefore, we might expect that either: (i) a luminal, secondary active transporter does not exist, or (ii) if it does exist, it functions in the acid-extruding direction when luminal pH is high, but shuts off when ion gradients are not favorable for acid extrusion.

The second question concerning pH_i regulation in "tight" epithelia is whether the electrogenic H^+ pump is responsible for regulation of pH_i in the mitochondria-rich or granular cells. If a proton pump were to serve the dual role of a urinary acidifier and a pH_i regulator, as the $Na-H$ exchanger appears to do in the renal proximal tubule, then one would expect the pump rate to be inversely related to pH_i. Cohen and Steinmetz (1980) have

measured proton pump currents in the bladder and compared these with pH_i values obtained by the DMO method on the whole bladder. Indeed, they found that the pump rate rises as pH_i falls. It is also of interest to note that a recently proposed model which accounts for the available data on the epithelial proton pump (Steinmetz & Andersen, 1982) also predicts that the pump rate should vary with pH_i. When the authors' values for the various rate constants are employed in their mathematical model, the predicted pump rate rises sigmoidally from about 2% of the maximal rate to about 98%, as pH_i is lowered by ~ 1.5 on an arbitrary pH scale (constant luminal pH). Both the data and this model thus suggest that the epithelial proton pump could function as a pH_i-regulating mechanism. The mitochondria-rich and intercalated cells could also possess a secondary active transport system for regulating pH_i. The same restrictions would apply to such a hypothetical system as discussed above in connection with the granular and principal cells.

It is instructive to briefly consider the evolutionary significance of proton pumps in vertebrate epithelial cells. In bacteria and fungi, not only is a H^+ pump a major consumer of metabolic energy, it is also responsible for establishing the H^+ electrochemical gradient which serves as the energy source of a variety of H^+-coupled secondary active transport systems. Thus, in addition to playing a role in regulating pH_i, the H^+ pump also controls a major portion of metabolism by energizing transport processes. Such an arrangement limits the cell's ability to regulate pH_i independently of metabolism. Inasmuch as pH_i is a crucial parameter in determining the activity of a wide variety of cellular processes, such a dual role for a H^+ pump has obvious disadvantages. In higher animals, the gradient-generating niche is now filled by the $Na-K$ pump. Thus, these cells are able to regulate pH_i relatively independently of numerous other transport processes, which are now coupled to the Na^+ and not the H^+ gradient. A question that arises in connection with mitochondria-rich and intercalated cells is whether the H^+ gradient generated by the H^+ pump at the luminal membrane also used to drive secondary transport processes.

IV. Future Directions

In the case of the secondary active transport systems, we are lacking a detailed thermodynamic and kinetic description. In particular, it remains to be firmly established that the $Na/HCO_3-Cl/H$ and $Na-H$ systems are driven solely by ion gradients. This would require a demonstration that the transporter can be put into equilibrium, and ultimately reversed, by the predicted combinations of ion gradients. Additional kinetic data are also needed, especially for the $Na/HCO_3-Cl/H$ system, to provide a more detailed picture of the ionic mechanism of the transport process. In the case of the epithelial proton pump, it would be of interest to determine how the pump rate depends upon various intracellular parameters, including pH_i, and to determine the role of this pump in pH_i regulation.

Future progress in the field will also depend upon the application of relatively new technologies. Although electrogenic proton pumps can be examined indirectly through their effects on current or voltage, this is not possible for the electroneutral systems. To date, progress on intact cells has been limited to those preparations which can be impaled by pH-sensitive microelectrodes. Although pH_i can also be measured by weak-acid and weak-base distribution methods, these are generally too slow to follow pH_i changes produced by transport systems or by passive fluxes of ionic weak acids and bases. As a result, such transport studies have been limited to a few rather large cells to which microelectrode techniques are applicable. However, two approaches show promise for use in even very small cells. The first is ^{31}P nuclear magnetic resonance. Although the machinery is expensive and requires special skills, this method can be used to obtain reliable measurements of pH_i on a fairly short time scale. The second promising technique is the intracellular use of pH-sensitive dyes, particularly those generated *in situ* (Thomas et al., 1979). Both approaches are reviewed in greater detail elsewhere (Roos & Boron, 1981). Aside from these techniques which can be applied to intact cells, the membrane vesicle technique has proven particularly useful. In the future, isolation and reconstitution of transport proteins may provide the ultimate description of the mechanisms of H^+ and HCO_3^- transport.

Recent work by a number of groups has implicated pH_i as a vital controlling factor in processes as diverse as cell division, activation of eggs and sperm, and mediation of hormonal activity. For a detailed description of these, the reader is referred to a recent symposium on the subject (edited by Nuccitelli & Deamer, 1982). If pH_i indeed proves to play an important role in regulating vital cellular activities, urgency will increase for achieving a detailed understanding of the transport of H^+ and of other ionic weak acids and bases in a wide variety of cell types.

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