Cellular mechanisms of $H^+$ and $HCO_3^-$ Transport in tight urinary epithelia

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When protons are produced during a variety of metabolic reactions they are eventually buffered by extracellular HCO$_3$. The task of the kidney in regulating acid-base balance is to regenerate the HCO$_3$ that is "fizzed" off. This is accomplished by proton secretion into the urine which results in generation of new HCO$_3$. Because of the original design of the kidney as a filtering organ, an additional task is the reabsorption of filtered HCO$_3$. This is achieved by the same process, H$^+$ secretion into the urine. This review will concentrate on the cellular mechanisms of H$^+$ transport in the tubular cell. In previous reviews (83), much space was devoted to the problem of the transported ion species, i.e. is it protons that are secreted or HCO$_3$ that is absorbed. We will not resurrect (exhume, is the more appropriate word) this issue below. The scope of this review will be largely limited to description of the processes involved in H$^+$ transport in the collecting tubule and urinary bladder. In essence, it will concentrate on the cell biology of transport by the intercalated cell of these tissues.

1. TRANSEPITHELIAL PROTON AND BICARBONATE TRANSPORT

H$^+$ and HCO$_3$ transport occur in two mammalian nephron segments, the proximal and collecting tubules. In amphibia and reptiles, the urinary bladder is an additional important site of acid/base transport as well. In this chapter we will discuss the processes of proton and bicarbonate transport in the collecting tubule and urinary bladder since the proximal tubule processes are covered in other chapters.

The epithelia that form the collecting tubules and urinary bladder have high transepithelial resistance, 500 ohm.cm$^2$ or greater. When these epithelia are exposed on both sides to media of identical composition they exhibit a transepithelial potential that is lumen negative with a value that may be as high as 100 mV. When transepithelial sodium transport is inhibited by ouabain (an agent that poisons the Na,K, ATPase) or amiloride (a blocker of the apical Na channel), the transepithelial potential declines and eventually reverses in sign (80). The cause of this reversed potential is the proton pump located at the apical membrane of the intercalated cells. The luminal contents of the turtle bladder or collecting tubule are acidified or alkalinized depending on experimental situations that will be described in detail below. These simple findings demonstrate that these epithelia absorb Na as well as transport protons and bicarbonate.

THE INTERCALATED CELL

The epithelium is composed of two cell types; a principal (or granular) cell and the intercalated cell (49,61,83,91). A third cell type, the basal cell is also found in some of these epithelia. The principal cell is the site of Na transport and responds to vasopressin by an increase in the permeability of the
apical membrane to water. The intercalated cell is enriched in mitochondria and carbonic anhydrase and is clearly the site of proton and bicarbonate transport. Using a monoclonal antibody to the Na,K ATPase, Kashgarian et al found no staining in this cell while the principal cells showed intense staining in the basolateral membrane (49). This finding need not imply that these cell do not contain the Na,K ATPase; the more likely explanation is that the density of the Na pumps is below the resolution of this method. The apical membrane of the intercalated cell shows many specializations when examined by the scanning electron microscope (46,61). Some cells have ruffles on their apical surfaces, (microplicae to the cognoscenti); others have microvilli. Detailed morphologic analysis has shown that this cell can exist in other forms. One of these forms in the rabbit has a peanut lectin binding protein on its apical surface which allows rapid identification of cells in sections or in perfused tubules (54).

**PROTON SECRETION**

Several methods can be used to measure secretion of protons into the lumen. In the isolated perfused tubule, the HCO₃⁻ concentration at the beginning and end of the tubule are measured and the fluxes can be calculated using the known perfusion rate (8,57,63,64,71-73). In epithelia mounted in Ussing chambers, one can use continuous titration of the luminal fluid while keeping the pH constant (pH stat method) (80,83). Alternatively, the spontaneous transepithelial potential is nullified using an external voltage clamp and the current needed to achieve this, the short circuit current can be continuously monitored (83). The short circuit current, a measure of net charge transfer across the epithelium, is due to two ionic movements, Na away from the lumen and protons towards the lumen. When Na transport is inhibited by amiloride or ouabain, the short circuit current is entirely due to proton secretion (9,83). Strictly speaking, this equivalence is expected only when there are no ionic gradients across the epithelium. In practice, however, this equivalence is present even when the media on either side are not identical. This is due to the fact that these epithelia are tight and do not allow significant diffusion potentials to develop.

That there is equivalence between proton transport and current flow implies that transepithelial proton secretion is "electrogenic". In the steady short-circuited state, this requires that current flow across the apical membrane, must equal current flow across the basolateral membrane. The model that satisfies these requirements is shown in Fig 1. The electrogenic elements here are the apically located proton translocating ATPase and the basolateral chloride channel. The Cl:HCO₃⁻ exchanger transports the two ions in a one to one fashion, hence, it does not participate in current flow. Recently Koeppen impaled cells from the medullary collecting tubule, which are likely to
be the acid secreting cells and found that they behave in an
electrophysiological manner that fits the above model (51,52). He
further demonstrated that the apical membrane does not contain
Cl, Na or K conductances, while the basolateral membrane contains
only a Cl conductance.

We see from the above that transepithelial proton secretion
is mediated by an ensemble of proteins that are deployed in two
membranes arranged in series; these include the proton
translocating ATPase, a Cl:HC03 exchanger, a Cl channel and
carbonic anhydrase. Each of these proteins is rate-limiting for
transport. An important aspect of the model is that the proton
pump is located in the apical membrane and that this membrane is
impermeant to protons. Early studies by Steinmetz had shown that
the cell pH of the turtle bladder was higher than that of the
luminal medium suggesting that the proton pump was located in the
apical membrane (80). Although the method used, in retrospect,
did not actually measure the pH of the intercalated cell (it
employed the distribution of DM0 in the whole epithelium
i.e. principal cells, smooth muscle as well as intercalated
cells), the conclusions were correct when more appropriate
methods for the measurement of cell pH in intercalated cells were
used. Another method was used by Dixon and Al-Awqati who showed
that imposing pH and potential gradients that were greater than
the reversal potential of the proton pump caused accumulation of
ATP in poisoned bladders indicating that the proton pump is an
ATPase that was located in the apical membrane (23). Gluck et al
demonstrated that apical membrane vesicles contained the proton
translocating ATPase (41). Finally, recent studies using
antibodies to the purified proton translocating ATPase showed
that there was staining of the apical membrane in the acid
secreting cell (12,76). These studies in the aggregate leave no
room for doubt regarding the location of the ATPase in the acid
secreting cell.

It is critical that the proton pump be located in a membrane
that is impermeant to protons (82). To demonstrate that we used
an indirect method whereby we measured the rate of glucose
oxidation simultaneously with the rate of transepithelial proton
transport (9). We found that reduction of the pH of the luminal
medium resulted in a reduction of the rate of proton secretion
and of the rate of glucose oxidation. This showed that the rate
of oxidative metabolism of the intercalated cell is determined by
the rate of ion transport. We performed similar experiments where
we changed the rate of transport by changing the ambient pCO2 at
constant transepithelial pH gradients. The ratio of net transport
to glucose oxidation in the presence of pH gradients was the same
as that determined in the absence of gradients. The ratio in the
absence of gradients is that intrinsic to the system since the
rate of transport is due only to the proton pump. On the other
hand, the proton current in the presence of gradients is, in
principle, due to the pump current minus the backflux into the
cell driven by the low luminal pH. The fact that the two ratios were identical showed that the proton current in both situations represented the pump current implying that even though the luminal pH was low there was no proton leak in the apical membrane. The finding that the apical membrane does not appear to contain any conductance further documents this finding (51).

The proton pump can be conceived of as an engine that splits water which then extrudes the protons leaving an excess of OH in the cell (Fig. 1). Carboxylation of the hydroxyls by CO₂ is facilitated by carbonic anhydrase. For transepithelial proton secretion to occur, the resultant HCO₃ must be translocated across the basolateral side. The mechanism by which this occurs was found to be exquisitely dependent on basolateral Cl concentration with a K1/2 of less than 1 mM (33). Removal of Cl from the basolateral medium alkalinizes the cell and its addition results in acidification implying Cl:HCO₃ exchange (1,73). Since the best studied anion exchanger is the erythrocyte band 3, investigators used the disulfonic stilbenes, SITS or DIDS, which inhibit the erythroid band 3 with high affinity. They found that this reagent inhibits the efflux of HCO₃ and therefore net transepithelial proton transport (19,30). As will be discussed later, there is now good evidence that the basolateral Cl:HCO₃ exchanger is either band 3 itself or a homologous protein. The high affinity for Cl suggests that it is different from the erythroid band 3 but more work needs to be done to document this point.

As mentioned above, Koeppen found a Cl conductance in the basolateral membrane of acid secreting cell (51). This Cl channel plays an essential in transepithelial transport since it disposes of the excess Cl that is brought into the cell by the Cl:HCO₃ exchanger. It is curious that the intracellular vesicles that carry the proton ATPase to the apical surface are known to contain Cl channels (37,42,93). Why the channels are not active in the apical membrane implies either that they are not inserted there or that they are inactivated once inserted.

HCO₃ SECRETION

Turtle urinary bladders and mammalian cortical collecting tubules are capable of urinary alkalinization as well as urinary acidification (8,11,36,55,57,53,64,66,69,72,73,78,83,86,88). That these two processes are independent of each other and can occur simultaneously can be demonstrated using maneuvers that specifically inhibit proton secretion. When the luminal pH of the turtle bladder was reduced to 4.5 the rate of proton secretion fell to zero. Addition of HCO₃ to the serosal medium resulted in net urinary alkalinization (55). This HCO₃ flux was not due to simple diffusion down the HCO₃ chemical gradient since it was inhibited by metabolic poisons and depletion of metabolic substrates. When glucose or pyruvate was added to these depleted
bladders there was stimulation of HCO$_3$ secretion (66). Further, replacement of Cl by sulfate or gluconate in the luminal medium resulted in inhibition of HCO$_3$ secretion. Mesurement of unidirectional chloride fluxes showed that addition of serosal HCO$_3$ resulted in stimulation of Cl absorption and that the Cl:HCO$_3$ stoichiometry was 1:1 (55). These results suggested that the turtle bladder was capable of secreting HCO$_3$ by an active transport process and that the mechanism of secretion involved a 1:1 exchange for luminal chloride. Similar results were obtained in the cortical collecting tubule (63,64).

Other studies demonstrated that the HCO$_3$ secretory flux shares some characteristics with the proton secretory flux. They are both inhibited by carbonic anhydrase inhibitors. Both types of fluxes are abolished in the absence of Cl; H$^+$ secretion on removal of basolateral chloride and HCO$_3$ secretion on removal of luminal chloride. Disulfonic stilbenes applied to the basolateral medium inhibit H$^+$ secretion but have no effect on HCO$_3$ secretion while luminal stilbenes inhibit HCO$_3$ secretion (46). These studies demonstrate that proton and bicarbonate secretion occur through two separate pathways that do not communicate with each other. The similarities between them further suggest that both fluxes occur through intercalated cells. More recent studies demonstrate that there are two types of intercalated cells, one subserving proton secretion while the other mediating bicarbonate secretion (73). This will be discussed in greater detail in another section of this chapter.

The cellular mechanism for HCO$_3$ secretion is now known with some certainty (Fig. 2, left). Proton ATPases are located in the basolateral membrane where they also participate in active endocytosis and exocytosis (73). The excess base generated by the action of the proton pump leaves the cell across the apical membrane by a Cl:HCO$_3$ exchanger that may be an analogue of band 3 (73). Osmotic balance must be achieved by a chloride channel. One interesting difference between the two fluxes is that proton secretion is "electrogenic" while HCO$_3$ secretion is electroneutral. Based on a simple equivalent circuit, the chloride conductance in the HCO$_3$ secreting cell must be on the same membrane as the electrogenic proton ATPase; i.e. the basolateral membrane. In such a configuration, there will be no net charge transfer across apical or basolateral membrane. Indeed, Koeppen has recently found that some cells in the collecting tubule have a basolateral chloride conductance. Based on the response of the membrane potential to carbonic anhydrase inhibitors, he concluded that these cells were intercalated cells. Although this is a reasonable conclusion and fits well with the predictions of the equivalent circuit for a HCO$_3$ secreting cell, we need to get intracellular electrical recordings in rigorously identified intercalated cells. It is interesting in this regard that treatment of turtle bladders (and possibly the cortical collecting tubule) with cyclic AMP,
phosphodiesterase inhibitors or calcium ionophores results in electrogentic HCO₃ secretion (31,71,86).

2. MOLECULES INVOLVED IN ACID/BASE TRANSPORT

A. PROTON TRANSLOCATING ATPase (2)

Proton transport across a membrane can be mediated by a variety of mechanisms. These include exchangers (e.g. Na:H or Cl:HCO₃), cotransporters (e.g. NaHCO₃ cotransport), or proton pumps. There are three fundamentally different classes of proton pumps; proton ATPases, redox mechanisms and photoactivatable proteins. All three types are widely distributed in nature and a single cell sometimes contains all three types of proton pumps. All cells contain at least one proton ATPase and one redox chain. To distinguish between a redox mechanism and an ATPase is relatively easy when one is using isolated membrane vesicles. If the vesicles are of the appropriate orientation, then external ATP should stimulate proton uptake in the case of an ATPase. For redox mechanisms an electron donor/acceptor pair can be added asymmetrically to the vesicles and proton transport measured.

Distinguishing between the two pump mechanisms is easy in isolated membranes but quite difficult in intact cells or in epithelia. To identify a critical step in a pathway one usually relies on measurement of the concentration of reactants and products. In the case of the proton ATPase, for instance, one can vary the rate of transport and measure the intracellular concentration of ATP, ADP and Pi. For the redox mechanism one can measure the concentrations of the likely redox pair i.e. usually NAD/NADH or NADP/NADPH. One problem with these methods is that critical reactants e.g. ADP, NAD or NADPH are shared by many cytosolic reactions. Hence, regardless of the actual pump mechanism, changing its rate will affect the rate of ATP hydrolysis as well as the rate of electron transport through redox pathways. Hence, the finding that the rates of oxygen consumption, glucose oxidation or the pentose pathway are coupled to transepithelial H⁺ transport is not evidence that the proton pump is an ATPase or a redox mechanism. It only shows that H⁺ transport is energy dependent and that cellular metabolism is tightly coupled.

Another approach that has been used is that of inhibitors that are supposed to be specific for one process or another. The problems of this approach is that the specificity of an inhibitor is difficult to demonstrate. In the final analysis, nothing substitutes for direct tests of the nature of the proton pump. The approach we initially took was based on the argument that the rate of transepithelial H⁺ transport is reduced when an adverse proton electrochemical gradient is imposed on the turtle bladder (3,9,82). We reasoned that that a gradient larger than the "reversal potential" of the proton pump should cause the proton
pump to "run backwards" synthesizing ATP if it was an ATPase or oxidizing the reduced intermediate of the redox pair if it was a redox pump. However, the ATP generating and consuming reactions of the cell will have to be inactivated before such a test will yield conclusive results. This again follows from the problem mentioned above that cellular metabolism is tightly coupled. Unfortunately, to inactivate these reactions one needs to use large doses of inhibitors which could inhibit the proton pump. Hence, a negative result yields no information. Further, inhibition of cellular metabolism could render the cell membranes leaky to protons, dissipating the external gradients that are imposed. Despite all these problems, Dixon and Al-Awqati were able to demonstrate accumulation of ATP in response to large gradients imposed across the apical membrane of the turtle bladder (24). The tissue was poisoned with iodoacetate and cyanide such that its ATP levels were down to near zero. Imposing pH and/or potential gradients resulted in ATP synthesis that was proportional to the gradient. Further when the cell was alkalinized by serosal HCO₃⁻ we obtained a result equal to mucosal acidification. These results provided strong evidence that the proton pump in the turtle bladder, and by analogy the terminal nephron, was a reversible proton translocating ATPase. When we added luminal inhibitors such as dicyclohexyl carbodiimide (DCCD) or oligomycin we abolished the rate of ATP synthesis. We interpreted these results to indicate that the ATPase was sensitive to DCCD and oligomycin. Subsequent experiment showed that while the ATPase was sensitive to DCCD, it was resistant to oligomycin. The reason for the discrepancy is likely to be due to the fact that adding another metabolic poison (DCCD or oligomycin) on top of iodoacetate and cyanide killed the cell making it leaky thereby preventing the development of the proton gradients needed to induce reversal.

A more direct approach was obtained by isolation of vesicles from the turtle bladder and kidney medulla which demonstrated that addition of ATP resulted in the acidification of the interior of the vesicles (41,42). It was also possible to demonstrate that the ATPase was electrogenic and to characterize its inhibitor profile. Gluck et al found that this proton ATPase is packaged in small endocytic vesicles located under the plasma membrane of the intercalated cell (40). Other studies demonstrated that endocytic vesicles of many cells are acidified by a proton ATPase which is similar to the renal one (2).

Proton translocating ATPases are membrane proteins that vectorially translocate H⁺ from one surface to the other (for a recent review see ref 2 from which some of the following has been taken). Studies on a variety of proton translocating ATPases (as well as other ion translocating ATPases) have uncovered sufficient detail to allow a broad classification of these ion pumps into two classes, an E₁-E₂ type and an F₀-F₁ type. A third type of ATPase occurs in eukaryotic cells and is located in the
plasma membranes as well as in a variety of intracellular organelles such as endosomes, golgi and endoplasmic reticulum. Although this recently discovered microsomal ATPase most likely represents an F₀-F₁ type the lack of detailed structural information prevents definitive assignment to one or another type. It will be discussed in detail below.

E₁-E₂ These proton pumps are present in yeast and fungal plasma membranes and the gastric microsomal and plasma membranes (43,68). This ATPase contains one large transmembrane protein (Mr ~ 100 kD) which contains both ion-conducting and catalytic domains. The E₁-E₂ family of ATPases also includes the Na,K ATPase and the Ca ATPase. Many, though not all of these ATPases catalyze cation exchange with a variety of stoichiometries, hence some of these pumps are "electrogenic", i.e. they are able to generate a membrane potential in the absence of any ion gradients. The gastric enzyme is a neutral K⁺:H⁺ exchanger, while the yeast and fungal enzyme is electrogenic. The enzyme is phosphorylated on an aspartyl residue during the catalytic cycle and dephosphorylation is necessary for completion of the cycle. One characteristic of these ATPases is that they are inhibited by vanadate, a transition metal anion which probably substitutes for phosphate resulting in stable vanadylation of the enzyme and consequent inhibition of the reaction. Based on the deduced amino acid sequence remarkable structural similarity was found among these enzymes; the Na,K ATPase Ca ATPase and yeast plasma membrane H⁺-ATPase are diverse enzymes whose structural similarity suggests common evolutionary ancestry. The most dramatic instance of conserved homology is in the sequence surrounding the aspartyl residue which is phosphorylated during the cycle. Proton ATPases which belong to this class of pumps have not been identified in the kidney.

E₀-E₁ These pumps only catalyze electrogenic proton transport (6,35). This enzyme is composed of two portions: a transmembrane portion (F₀) which acts as a proton channel and a catalytic portion (F₁) that can be easily released from the membrane. The F₀ domain is composed of as many as 3 proteins one of which is clearly a proton channel (subunit c, the proteolipid, or DCCD-binding protein) while the others probably function to stabilize the channel structure and to allow binding of F₁. The conductance of F₀ purified from thermophilic bacteria and reconstituted into liposomes increases as a function of the H⁺ concentration suggesting that the species of ion conducted is the proton rather than OH-. The large catalytic domain is composed of at least 5 proteins; the alpha and beta subunits contain the nucleotide binding and hydrolysing sites. Using electron microscopy and negative staining it can be shown that the F₁ domain protrudes as a sphere above the bilayer surface to which it is connected by a stalk. This "lollipop" appearance is characteristic of these proton pumps. The enzyme is not phosphorylated during the catalytic cycle and no occluded state has been discovered. Except for those isolated from bacteria, this enzyme
has not been completely sequenced. From the prokaryotic studies, the nucleotide binding domain appears to have extensive homology to other ATP-requiring or utilizing proteins such as adenylate kinase, tRNA synthetase, recA protein and RNA polymerase. In eukaryotes, where these proton ATPases are present in mitochondria and chloroplasts, the genes coding for most of the subunits are present in nuclear DNA rather than the mitochondrial or chloroplast DNA. Although the gene for the proteolipid is in the mitochondrial DNA in one species (yeast), it is located in the nuclear DNA in Neurospora. The proteins are synthesized in some instances on free ribosomes and in others on ribosomes bound to mitochondria and the proteins are then cotranslationally inserted into the mitochondria.

**Microsomal ATPase, including the renal proton pump**

Although it had been known for some time that the contents of lysosomes are acid it is only recently that this organelle and many others were found to be acidified by proton translocating ATPases. These proton pumps are present in endosomes, secretory granules, golgi, endoplasmic reticulum as well as in the plasma membrane of many eukaryotic cells. They are also present in yeast vacuoles and plant vacuolar membranes. The lack of sensitivity to vanadate suggests that it does not belong to the E₁-E₂ type of enzyme. None of these enzymes have been purified to homogeneity; however, preliminary results suggest that this enzyme is a multisubunit complex composed of possibly 8 proteins, the smallest subunit bound ^14^C-DCCD suggesting that it might be an analogue of the proteolipid of the F₀-F₁ ATPase (94). Experiments using kidney medulla suggests that the proton pump there has at least 5 subunits with molecular weights of 70, 56, 44, 35 and 17 kDa (38). They also found "lollipop" type images on negative staining (12). The finding that this ATPase is a multisubunit protein one of whose subunits binds DCCD provides evidence, albeit indirect, that this enzyme belongs to the F₀-F₁ class of proton pumps. However, it will be necessary to demonstrate that this complex contains a proton conducting portion that is an integral membrane protein(s) and a catalytic domain which can be released from the bilayer by mild treatment. One would expect that the catalytic portion can bind to the proton conducting domain and occlude it thereby reducing proton permeability. One of the important questions in this area is whether the proton pumps in the various organelles are the same enzyme or are simply similar proteins. So far they all appear to be similar in their drug sensitivities. It had been difficult to demonstrate that the lysosomal ATPase is electrogenic, but that is probably due to the fact that lysosomal membranes are electrically "leaky". It also appears that the lysosomal ATPase can use GTP as well as ATP for pumping protons; the chromaffin granule enzyme can also use ITP, though less well than ATP. If this turns out to be true in the purified enzymes, it implies that there are related types of the microsomal ATPase which can be separated on the basis of their
nucleotide specificity.

Morphological studies of the intercalated cell in urinary bladder and collecting tubules have shown that the endocytic vesicles of these are enriched in "studs" (13,14,86). These structures which can be seen on freeze fracture electron microscopy are located on the cytoplasmic face of the apical plasma membrane of acid secreting cells as well in the endocytic vesicles. Recent studies by Brown and Gluck have shown that these studies are the proton ATPase of these vesicles (12). When the purified proton ATPase was reconstituted into lipid vesicles and examined by freeze fracture, studs of similar size and morphology were found in the liposomes. Previous studies by Stetson had not found these studs on golgi or lysosomes even though these structures clearly contain proton pumps which raises the question that either the density of the proton pumps in these organelles is low or that the ATPases are fundamentally different (86).

Another characteristic of the proton ATPase that needs to be established is the H/ATP stoichiometry of the pump (\( \eta \)). The stoichiometry of an ion pump is best studied by measuring the rate of ion transport simultaneously with the rate of ATP hydrolysis. This has not been done in the renal ATPase, for it poses a large technical problem. In lieu of such measurement Steinmetz et al measured the rate of lactate production before and after stimulation of \( H^+ \) transport in anaerobic bladders (81). There was good coupling between the two rates and the ratio of the two fluxes was near 2.0. Another approach used the ratio of the driving force, i.e. the free energy of ATP hydrolysis (\( \Delta G_{ATP} \)) to the maximum electrochemical gradient generated by the pump (\( \Delta \mu_H \)) and found the ratio to be 3- (23).

\[
\eta = \frac{\Delta G_{ATP}}{\Delta \mu_H}
\]

There is now reasonable agreement that the \( \Delta G_{ATP} \) of cells is at least 14 kcal/mol (59 kJ/mol or 600 mV) (90). When one interpolates the maximum electrochemical gradient of 3 pH units or 180 mV, one obtains a stoichiometry of 3.4 H/ATP. A recent kinetic model of the proton ATPase proposed by Andersen et al suggests that the maximum electrochemical gradient is frequently underestimated by as much as 1 pH unit which would reduce the stoichiometry to values of 2.5 or so (7,79). However, given the uncertainty of what is actually the maximum electrochemical gradient, and whether lactate production is an actual measure of the rate of ATP hydrolysis, the numerical value of the H/ATP is at present not clear. Its scientific value, however, is unquestioned, since it will be impossible to achieve a detailed knowledge of the actual kinetic mechanism of the proton pump without this crucial piece of information.

B. Cl:HCO₃ EXCHANGE
For transepithelial transport to proceed, the HCO$_3^-$ generated by the proton ATPase has to exit across the plasma membrane that is opposite to that which contains the proton ATPase. This exchanger was identified as a consequence of the finding that transepithelial proton secretion was dependent on basolateral chloride and the earlier finding that transepithelial HCO$_3^-$ secretion was dependent on luminal chloride (36,55,73,78,88). The dependence was found to be exquisite with apparent half maximal concentrations of less than 1 mM (33). Studies of intracellular pH in whole epithelia and in identified intercalated cells showed that removal of chloride from the basolateral side of acid secreting cells prevented the exit of HCO$_3^-$ resulting in cellular alkalinization (73). In HCO$_3^-$ secreting cells, removal of basolateral chloride caused an increase in HCO$_3^-$ secretion and cellular acidification. Although cellular chloride concentrations have not been measured as a function of changes in external or internal HCO$_3^-$, these results provide reasonable evidence for the presence of a Cl:HC0$_3^-$ exchange in plasma membranes of the intercalated cells. This process is inhibited by disulfonic stilbenes, DIDS and SITS (18,30). Whether the apical and basolateral Cl:HC0$_3^-$ exchangers are the same protein is not yet clear.

Recent studies from several groups have identified the basolateral exchanger of the acid secreting intercalated cell, as either the erythroid band 3 or a homologue of it (28,71). Band 3, the most abundant integral membrane protein in erythrocytes, has long been known to mediate the exchange of Cl for HCO$_3^-$, a process that is critical for CO$_2$ transport in blood (48). This protein, M.W. of approximately 100kDa, is composed of two domains; the N-terminal cytoplasmic domain includes binding sites for a number of cytoskeletal proteins. It appears that band 3 serves as an organizing "focus" for the red cell skeleton composed largely of spectrin and actin which is responsible for maintenance of the red cell shape (10). The C-terminal domain spans the membrane several times and contains the anion transport site. Cleavage of the N-terminal domain has no effect on ion transport. DIDS binds to a lysine (Lys 561 or 568 in the murine sequence) which is accessible from the extracellular surface. The recent cloning of the cDNA for murine erythroid band 3 (53) has allowed the detection of messages in kidney and liver cells which code for a band 3 homolog (5,22). This new species has extensive homology with the membrane spanning domain of erythroid band 3 but the homology with the N-terminal, cytoskeletal binding domain is quite low. Whether this is the only homolog is unknown. Many cells contain Cl:HC0$_3^-$ exchangers which conceivably could all be members of one gene family. Whether this putative family also contains members that code for chloride channels is possible but unlikely.

Studies at the protein level have also identified proteins in kidney homogenates that react with antibodies to band
3. Studies by Drenckhahn et al have shown that rat kidney contains a cross reacting material that has a molecular weight of 120 kDa (28). In chick kidney the cross reacting material was 140 kDa (21). Although it is likely that these proteins are homologs of band 3, it is still too early to comment on the closeness of their kinship. Antibodies generated to band 3 were used by several groups for immunocytochemical localization. Only the intercalated cell showed any staining. These antibodies include polyclonal anti-human band 3 as well as monoclonal anti-human band 3. In our studies we used an antibody generated by Kopito and Lodish to the C-terminal peptide of the murine band 3 (Herzlinger and Al-Awqati, unpublished results).

C. CHLORIDE CHANNEL

Although there is no biochemical evidence for the presence of a chloride channel in intercalated cell, the a priori electrophysiology of these cells mandates its presence. When protons are pumped out of the cell at one surface, the excess HCO₃ leaves the cell at the other surface, but only in exchange for Cl. Hence, for maintenance of osmotic balance during transepithelial proton and HCO₃ transport, it is necessary that another chloride transporting mechanism be present. That this an electrogenic mechanism, i.e. a channel, is implied from the fact that transepithelial proton secretion is electrogenic and is produced by a proton ATPase known to be electrogenic and a Cl:HCO₃ exchanger which is known to be neutral. For transepithelial current flow to occur, the Cl channel must be located on the basolateral membrane in the case of the H⁺ secreting intercalated cell. Since, at least in the turtle bladder, transepithelial HCO₃ transport is neutral, the chloride channel must also be on the basolateral side in these cells as well. Recent studies by Koeppen have suggested that in the inner stripe of the outer medullary collecting tubule (the site of the H⁺ secreting cells) there is no apical Cl conductance (51) while in the outer stripe (the site of the HCO₃ cells) there is a basolateral chloride conductance (52). Although it is difficult to identify the cells which have been impaled as being intercalated cells, let alone which subtype they are, the fact that the results fit with the predictions of the appropriate equivalent circuit discussed above suggests that his conclusions are correct.

It is only recently that electrophysiologists have been able to study renal cells with the patch clamp method. Greger has now found an epithelial chloride channel which has a single channel conductance of approximately 20 pS and shows reasonably linear current-voltage relationship (70). This channel was found in the basolateral membrane of the thick ascending limb cell. Whether the same or a similar channel is found in the intercalated cell remains to be demonstrated. Chloride channels exist in many endocytic vesicles which contain the proton ATPase, however,
their electrophysiology is not well characterized. Whether these channels are the same as the ones in the basolateral membrane of intercalated cells is an intriguing question since it raises an important issue in the segregation of membrane proteins from the proton ATPase vesicles to one or another epithelial plasma membrane domain in the two cell types.

Recent studies have shown that addition of calcium ionophores or cyclic AMP to tight epithelia stimulates the electrogenic secretion of $\text{HCO}_3^-$ (30,86). The most likely explanation for this finding is the one given by Steinmetz's group, which is the opening of an apical chloride channel by these reagents. This would result in recycling of Cl across the apical membrane and electrogenic $\text{HCO}_3^-$ secretion. Whether this new channel can conduct $\text{HCO}_3^-$ as well as chloride cannot be determined without direct electrophysiological testing. The mechanism of action of cyclic AMP is also of interest since it was not clear whether cyclic AMP opened channels that were resident in the membrane or caused insertion of new chloride channels. If the latter turns out to be the case then it implies chloride channels are present without any polarity but that polarized transport is induced by local activation or inactivation of the channel proteins. Cyclic AMP activates chloride channels in the apical membrane of tracheal mucosa and other epithelia (32,34,44,92).

D. CARBONIC ANHYDRASE (see Ann. N.Y.Acad.Sci. v.429,1984 for a comprehensive review)

Carbonic anhydrases (CA) are the product of a multigene family that are widely distributed in animals and plants. There are at least four isoenzymes which are expressed in a specific manner in various tissues. Carbonic anhydrase I, II, and III are soluble enzymes that are present in the cytoplasm while CA IV, is a membrane bound enzyme. The first three isoenzymes are well characterized while the sequence of the fourth is not yet available. It is also now known that mitochondria contain carbonic anhydrase but its complete sequence is unknown.

All carbonic anhydrases are metalloenzymes which contain one atom of zinc per molecule. The zinc is chelated by three histidines in the active center, these are His 94,96 and 119 in human CAI and II. One hydroxyl is also coordinately attached as well as a solvent molecule which forms hydrogen bonds with, in all likelihood, Thr 199. The pentacoordinated zinc is located in a pocket of the enzyme that has hydrophobic as well as hydrophilic amino acids. Carbonic anhydrase is involved in the hydration of $\text{CO}_2$ and in its dehydration. The uncatalysed reactions are

$$\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \quad k = 0.035 \text{ /sec}$$

$$\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^- \quad k = 8500 \text{ /sec}$$
Catalysis cannot proceed by acceleration of the first reaction since the supply of carbonic acid (the substrate in that reaction) is limiting. At the pH range where carbonic anhydrase is active, the concentration of carbonic acid is very low and its supply by protonation of HCO$_3^-$ cannot account for the known turnover number of the enzyme. The second reaction is fast but only at pH's greater than 10. The most likely mechanism is that the hydrophobic pocket where the zinc is located reduces the pKa of coordinated water and generates a coordinate hydroxyl. This mechanism explains the marked effect of anions on the activity, where the added anions compete with the OH$^-$ in the formation of the ZnOH$^-$ complex (20). The final reaction can be written as follows:

$$\begin{align*}
-H^+ \\
\text{Zn.OH}_2 \rightarrow \text{Zn.OH}^- + \text{CO}_2 \rightarrow \text{Zn.HCO}_3^- + \text{H}_2\text{O} \rightarrow \text{Zn.OH}_2 + \text{HCO}_3^- + \text{H}^+
\end{align*}$$

Although carbonic anhydrases catalyse the CO$_2$ hydration and dehydration reactions they also catalyse many other reactions. They are potent esterases and enzymes that catalyse the hydration of aliphatic aldehydes and they have other activities as well. However, the rate of catalysis is much lower than that for the CO$_2$ reactions by several orders of magnitude suggesting that the latter is the more likely physiological reaction. CA II is the most active of the enzymes while CA I has a low turnover.

Carbonic anhydrase is widely distributed in mammalian tissues where in many instances its role in acid/base transport is quite obvious e.g. in the kidney tubule turtle bladder and gastric parietal cell (27, 67). The role that erythrocyte carbonic anhydrase (CAII) plays in CO$_2$ transport is well known, but it is now clear that carbonic anhydrase bound to endothelial cells of the lung and possibly elsewhere (CA IV) is also important in CO$_2$ transport. The role that carbonic anhydrase plays in osteoclast function has recently received some attention when it was discovered that patients with congenital osteopetrosis have a defect in the CA II gene and have renal tubular acidosis as well (89). In many secretory cells of the intestine, choroid plexus, sweat, salivary and mucous glands its role in secretion is less clear, but many of the secretions of these glands are either acid or alkaline. It is also located in muscle (CA III), oligodendrocytes in the brain, in taste buds, olfactory epithelia, and other areas where its role is quite obscure.
3. REGULATION OF PROTON SECRETION

A. Tranmembrane electrochemical gradient

Ion transport processes, even those fueled by ATP, respond to the electrochemical gradient across their membranes. The proton translocating ATPase, is no exception. Being electrogenic it responds to both the chemical gradient as well to the membrane potential. Initial experiments showed that the rate of H⁺ transport across the turtle urinary bladder was inhibited when the luminal pH was reduced, approaching zero when the pH reaches 4.5 (3,9,82). The well-known physiological and clinical observation that the minimal urine pH is near 4.5 is simply a reflection of the fact that the urine pH is largely determined by the ability of the proton ATPase to reduce the luminal pH down to its "reversal potential". The decline in the rate of transport was reasonably linear. Similar studies were performed relating the rate of transport to the trans-epithelial membrane potential at constant luminal pH. It was found, that there was also a linear decline in the rate of transport when the epithelium was depolarized (3). The surprising finding was that the slopes of the decline in response to the two moieties of the electrochemical gradient was identical. This identity would have been expected in a passive ion channel, its presence in a complex proton translocating ATPase suggests that the effect of pH on the rate of turnover of the enzyme was exerted on a segment of the protein that could not discriminate between an electrical and a chemical gradient. Recently Andersen et al analysed the kinetics of proton transport in response to imposed transepithelial electrical and concentration gradients (7). They found that the maximum rate of proton transport became independent of pH or membrane potential at high, and also at low rates of transport. They developed a kinetic model based on a scheme of the proton pump composed of two sectors, a proton channel and a catalytic segment and that he latter segment senses the proton concentration in an "antechamber" located between it and the channel. The model was able to predict the saturable behavior of the pump. It also led to the prediction that the reversal potential of the pump is larger than that measured usually, i.e. larger than 3 pH units or 180 mV. It is difficult to verify this latter prediction since the rates of transport in this range of adverse electrochemical gradient is so low as to be near the detection limits of the measurements.

The effect of electrochemical gradients on proton secretion is an important physiological regulatory step not only for transepithelial proton transport but also for proton pumping in intracellular vesicles and plays a significant role in the energy economy of cells. In renal transepithelial transport, the number of protons pumped can be kept at a maximum level by increasing the buffering power of the urine. Increased production of buffers that are permeant weak bases will increase the urine pH which, in
turn, will accelerate the rate of proton pumping. This mechanism is the basis for the important roles that NH₃ and other urinary buffers play in net acid excretion. Without these buffers it would be impossible to excrete the necessary net acid load.

Many intracellular vesicles are acidified by a proton translocating ATPase, similar or identical to the one responsible for transepithelial H⁺ transport. The effect of the pH gradient and the membrane potential on the rate of transport is similar to that on transepithelial transport. This is illustrated in Fig. 3, which shows the development of pH gradient and membrane potential in golgi vesicles obtained from rat liver (37). The development of a pH gradient is measured by the uptake of a weak base, acridine orange, while the development of a membrane potential, positive inside is followed as the ejection of the cationic carboxyamine dye, di-S-C3(5). When valinomycin, an electrogenic K⁺ ionophore is added to the vesicles the rate of proton transport is accelerated. This implies that when the membrane potential, positive inside is collapsed, the proton pump can pump faster. When a pH gradient is collapsed by a neutral K⁺ exchange, nigericin, the membrane potential is increased due to the fact that the the pump is electrogenic and will generate a larger membrane potential when its turnover is accelerated. Hence, in these vesicles, the steady state is associated with the development of the maximum chemical or electrical gradient. At that point, the rate of pump turnover is minimal, and is simply related to the rate of leakage of protons or electric charge from the vesicles. This clearly is a most energy efficient process where these vesicles after an initial burst of ATP hydrolysis, reduce their hydrolytic activity by this "autoregulatory" mechanism.

B. Cell pH

Since protons can be thought of as substrates for the proton translocating ATPase one would expect to find that reduction of cell pH will result in an increase in the rate of proton translocation. Indeed, there is good evidence that reduction of cell pH is associated with stimulation of proton pumping. An increase in the ambient pCO₂ causes a stimulation of H⁺ secretion in turtle bladder, a maneuver that reduces cell pH (1,19,74,83). Further, addition of weak acids stimulates H⁺ secretion in the turtle bladder. What is less clear is whether the reduction of cell pH has an effect directly on the proton ATPase to stimulate its turnover or whether the effect is more complex. As will be described below, there is now strong evidence that reduction in cell pH by any maneuver, causes an increase in cell calcium which induces exocytotic insertion of more proton ATPases into the membrane. Is there any effect of lowering the cell pH on the kinetic behavior of individual proton pumps? The effect of pH on enzyme activity has been measured and found to show a flat "pH optimum"; there was little change in activity
between pH 6.5 and 7.5 (41). Hence, it is likely that there is no intrinsic effect of cytosolic pH on the activity of the individual proton pumps. It would be important to test the effect of pH on proton transport in vesicles. This will likely to be a difficult problem since one ought to test this issue without any change in the pH gradient. Since many of the available methods use the distribution of weak bases, and since the pH of the vesicle contents is likely to determine its buffering power, the results of such experiments will likely be qualified rather than decisive. Although lowering pH had no effect on the proton ATPase enzyme activity, we have not ruled out some complex covalent modification, e.g. by phosphorylation, which could be induced by lowering the cell pH and which requires cytosolic factors for its expression. As will be discussed below, addition of CO₂ acidifies the cell which leads to an increase in cell calcium. The latter causes exocytotic insertion of proton ATPases which then bring the cell pH towards the initial level. When calcium was prevented from increasing, there was no exocytosis and the cell pH remained low. We interpret these experiments to show that there is little or no effect of cytosolic pH on the activity of the proton ATPase within the usual physiological range. Rather, the sole effect of lowering cell pH is to cause exocytotic insertion of proton ATPases.

C. The effect of CO₂ and the role of Exocytosis

An increase in the ambient pCO₂ stimulates H⁺ secretion while a decrease inhibits it. The effect of CO₂ is saturable in the turtle bladder, showing no further increase above a concentration of 5% in air (74). No similar titration is available in the mammalian tubule. These results suggest that if the same titration curve applies to mammals, the effect of CO₂ will be expressed in hypocapnia. Initial studies showed that increasing the ambient pCO₂ increased the rate of transport without changing the maximum gradient that the proton pump can generate. These results could be due either to an increase in the number of proton ATPases on the surface or to a change in the kinetic parameters of individual proton pumps. We initially found that the intercalated cells are enriched in vesicles that were acidified by the proton ATPase (15,40). These vesicles were constantly fusing with the apical plasma membrane only to reform as endocytic vesicles. Using this property we were able to load these vesicles with fluorescent macromolecules that allowed us to measure the pH in these vesicles. Further, by passing the luminal solution into a fluorometer we were able to monitor any secretory event that might occur. While simultaneously measuring the rate of trans-epithelial proton transport (using the short-circuit current method in ouabain treated turtle bladders) we increased the pCO₂ in the basolateral medium. This caused an immediate rise in the rate of H⁺ transport, an increase that was persistent, Fig 4 top panel. This was accompanied by a transient secretion of fluorescent macromolecules (Fig 4 second
Since the fluorescent macromolecules were internalized into acid vesicles which then fused with the apical plasma membrane, it follows that an increase in pCO$_2$ caused exocytosis of vesicles which resulted in insertion of proton ATPases into the apical membrane. There was good correlation between the volume of fluorescent material secreted (an index of the number of vesicles inserted) and the increase in the rate of H$^+$ transport. Inhibition of exocytosis by colchicine also caused an inhibition of the increase in H$^+$ secretion. That the exocytosis inserted functional proton pumps into the apical membrane is shown by the fact that the cell pH recovered following the addition of CO$_2$, Fig 4 bottom panel (1). When these experiments were repeated in the cortical and medullary tubules of rabbit kidney we found identical results using modifications of the methods described for the turtle bladder (75). Morphometric studies also showed that there was a reduction in the volume of vesicles in the subapical region of these cells in both turtle bladder and collecting tubule.

Since it was very likely that the effect of CO$_2$ is produced by a lowering of cell pH, we tested the effects of butyrate or acetate and found that they, also, caused an increase in H$^+$ secretion and simultaneous exocytosis. The mechanism by which cell acidity could cause exocytosis was investigated by measurement of intracellular calcium (15). We found that acidifying the cell by either CO$_2$ or butyrate caused a transient increase in cell calcium, Fig 4 third panel. This did not occur when extracellular calcium was removed suggesting that calcium was coming from the outside rather than from intracellular stores (1). Preventing the increase in cell calcium by increasing the calcium buffering power of the cytoplasm or by removing extracellular calcium prevented the exocytosis and the increase in H$^+$ secretion (15). These results show that the proximate signal is cell acidification and the distal signal is calcium induced exocytosis. How lowering cell pH increases cell calcium remains to be determined. Since calcium seems to be entering the cell from the outside, it is likely that these cells contain calcium channels. Such channels have been found to be voltage regulated. Hence, one hypothesis worth investigating is that cell acidification might depolarize the membrane which would then open calcium channels. The influx of calcium will cause exocytosis which will return the cell to its original pH resulting in membrane hyperpolarization. It is well known that many potassium channels are pH-sensitive, closing when the cell pH is lowered. However, it has been difficult to obtain direct evidence for this, or any other hypothesis since these cells are difficult to identify and impale with microelectrodes.

In summary, the role of CO$_2$ and cell acidification is to increase the number of proton pumps in the apical membrane of the H$^+$ secreting cells rather than simply to accelerate the turnover of individual proton pumps. Recent studies have also identified
this mechanism in the proximal tubule where we found that there were also endocytic vesicles whose contents were acidified by a proton pump. Increasing the ambient pCO₂ also caused exocytosis of these vesicles. The role this exocytotic event might have in the renal response to respiratory acidosis is still unclear. It remains to be seen whether in mammalian kidneys an increase in ambient pCO₂ above 40 mmHg can cause an increase in the rate of secretion in the collecting tubule.

D. The role of endocytosis

When collecting tubules or turtle bladders are exposed to fluorescein-labelled dextran they internalize these macromolecules into acid vesicles. This demonstrates that there is continuous endocytosis of proton pumps from the luminal membrane. Since in the steady state the proton transport rate is constant, this implies that there is also continuous exocytosis of what has been endocytosed. We can term this constitutive endocytosis and exocytosis, whereby there is vesicle recycling at the luminal membrane. As pointed out above, acidifying the cell causes a wave of exocytosis that is regulated by changes in cell calcium. Recent studies have also identified a regulated pathway for endocytosis. In these studies the rate of internalization of FITC dextran was found to be stimulated when turtle urinary bladders were treated with inhibitors of carbonic anhydrase or when CO₂ was removed from the medium. These studies suggest that cellular alkalinization stimulates endocytosis. Further, Dixon et al found that when the bladders were treated with acetazolamide there was a concomitant decline in the surface area of the apical membrane as measured by impedance analysis (25). In this method the capacitance of the bladder is measured in situations that will minimize the contribution of the apical membrane of the principal cells. (Using this method these authors have previously found that CO₂ increases the apical membrane area) (17). These results are intriguing, but we need to know whether the vesicles that internalize the FITC dextran during the stimulated endocytosis actually contain proton pumps. (That the FITC dextran ends up in acid vesicles does not bear on this question since the dextran could rapidly be delivered to acid vesicles). These investigators recently used azide, a metabolic poison known to inhibit endocytosis (among other things) to probe this question. They found that azide does not affect the rate of H⁺ secretion in the bladder in the control state, but, it prevented the decrease in the rate of transport induced by acetazolamide. Azide also prevented the effect of acetazolamide in stimulating endocytosis and decreasing in apical membrane capacitance (26). These results demonstrate that acetazolamide and presumably cellular alkalinization stimulate the rate of endocytosis.

It is interesting to note that the time course of regulated endocytosis is quite different from that of regulated
exocytosis. In the latter process, the whole phenomenon is complete within a minute or so. The stimulated endocytosis is much slower although detailed kinetics are not available. These results raise a number of interesting issues. Of primary significance is the determination whether the exocytic vesicles are the same as the endocytic vesicles. In other words, when vesicles fuse with the apical plasma membrane, do the membrane proteins of the vesicle diffuse and mix with the other constituents of that membrane or do they remain "frozen". If they mix, what is the mechanism that selects for their presumably highly specific retrieval. Another set of questions concerns the relation between the constitutive and the regulated pathways. Are they the same, i.e. do changes in cell pH simply change the rate of constitutive exo-endocytosis or are there two parallel pathways for each process. Clearly molecular tools will need to be developed such that one can follow each of these groups of vesicles separately. Much remains to be done in this interesting area.

E. Aldosterone

Although there has been little doubt that aldosterone stimulates urinary acidification in the collecting tubule, it had always been assumed that the effect is secondary to stimulation of Na absorption. In the open-circuit state, increased Na reabsorption will hyperpolarize the epithelium and thereby stimulate H⁺ secretion down its electrochemical gradient. There is no doubt that this, indeed, occurs. However, the studies of Ludens and Fanestil, showed that aldosterone stimulates H⁺ secretion in the toad urinary bladder in the short-circuited state (60). Other similar studies in the turtle bladder and the collecting tubules documented the direct effect of aldosterone on H⁺ transport (41). Unfortunately, there is little detailed information on the mechanism of action of aldosterone on H⁺ secretion. The dose response curve in the toad bladder was similar to that for stimulation of Na transport but there appear to be important differences. Dexamethasone stimulates H⁺ secretion, but has no effect on Na transport. Spironolactone is a potent antagonist of the effect of aldosterone on Na absorption, but seems to stimulate H⁺ transport (65). Inhibitors of RNA and protein synthesis completely prevent the effect of aldosterone on Na transport but not on H⁺ transport. Further, these inhibitors, by themselves, stimulate H⁺ secretion suggesting that endogenous inhibitors might exist which are tonically suppressing proton transport (59). These findings raise a number of questions regarding the receptor which mediates the effect of aldosterone on proton transport. The distinction between glucocorticoid and mineralocorticoid receptors in the stimulation of Na transport in the toad bladder has not been as clear cut as elsewhere. However, the difference from the "canonical" behavior were relatively minor. In the case of H⁺ transport, the results do not fit any one model well. Whether it represents a new kind of receptor or
some modified mineralocorticoid or glucocorticoid remains to be determined.

F. Extracellular acid/base changes

Chronic acidosis is associated with an increased capacity of the kidney to excrete acid. This adaptive change occurs at different levels including proton transport by the collecting tubule and turtle bladder. The mechanisms by which this adaptation occurs is likely to represent an increase in the number of acid secreting cells in these epithelia. In the rabbit tubule this has already been demonstrated and will be discussed in detail in the section on epithelial polarity of proton and bicarbonate transport below. What is not known is whether the rate of transport in each acid secreting cell is increased. Another important question is whether chronic acidosis stimulates the synthesis of proton ATPases; an issue that is now approachable with the development of the appropriate antibodies.

G. CYCLIC AMP

Addition of cyclic AMP or inhibitors of its phosphodiesterase to the turtle bladder results in inhibition of the rate of transport (56). Whether a hormone exists that can cause this is unknown, but does not seem to be physiologically important since the changes are small. Since cyclic AMP stimulates electrogenic HCO₃⁻ secretion, it is possible that the inhibition of proton secretion seen may simply reflect the simultaneous increase in electrogenic bicarbonate movement rather than being an effect on proton transport itself.
4. REGULATION OF BICARBONATE SECRETION

Although there are a number of factors that regulate bicarbonate secretion, the amount and level of information is on a smaller scale than that for proton secretion.

A. Extracellular acid/base changes

When proton secretion in the turtle bladder was inhibited by lowering the luminal pH and the basolateral HCO₃ was increased a HCO₃ secretory flux was observed (55). The appearance of the secretory flux when the basolateral HCO₃ was increased implies that this flux is responsive to changes in the blood HCO₃ concentration. This is somewhat difficult to reconcile with our notions of the mechanism by which HCO₃ is secreted. There are proton ATPases and chloride channels on the basolateral membrane of these cells (Fig 2). How could changes in the HCO₃ concentration in that medium result in stimulation of transepithelial transport. One possibility is that the proton ATPase results in acidification of a basolateral unstirred layer sufficiently that the rate of the pump will decline; addition of HCO₃ will then titrate the unstirred layer back to an alkaline pH allowing the proton pump and net transepithelial transport to increase. Alternatively, the chloride channel could have a significant HCO₃ conductance, hence increasing the basolateral HCO₃ concentration will increase the cellular concentration with consequent stimulation of Cl: HCO₃ exchange. It is interesting that a recent preliminary study in the cortical collecting tubule seemed to indicate that an increase in the basolateral HCO₃ has no effect on HCO₃ secretion (11). Clearly more work needs to be done to resolve this problem.

The direction of bicarbonate transport in the cortical collecting tubule can be affected by feeding the animals an acid or an alkaline diet. When rats are treated with mineralocorticoids and simultaneously given a high intake of NaHCO₃ the rate of HCO₃ secretion increases (36). The secretory flux exhibits all the characteristics of HCO₃ secretion described previously in turtle bladder and rabbit tubule in that it occurs in strict exchange for luminal chloride. Whether there is a role for the mineralocorticoids in the stimulation of this secretion is not clear at present.

B. Cyclic AMP

Addition of cyclic AMP or inhibitors of cyclic AMP phosphodiesterase causes stimulation of HCO₃ secretion in the turtle bladder (29,87). This secretory flux, unlike the basal flux is accompanied by an increase in the short-circuit current. The best explanation for these results has been offered recently by Stetson et al where they demonstrate that the increase in secretion is most likely due to opening of apical chloride channels. It is interesting that cyclic AMP increases
the apical chloride conductance in other epithelia such as trachea, small intestine, colon and shark rectal gland (16,32,34,44,92). Whether this is the same chloride channel remains to be determined. In the turtle bladder opening an apical chloride channel will result in recycling of chloride across the apical membrane and secretion of \( \text{HCO}_3^- \) down its concentration gradient. The cell [\( \text{HCO}_3^- \)] is likely to be high due to the action of the proton translocating ATPase. Whether these chloride channels also conduct \( \text{HCO}_3^- \) remains to be determined. What is the physiological role of this mechanism? Brodsky has recently found that vasoactive intestinal peptide (VIP), increases \( \text{HCO}_3^- \) secretion in the turtle bladder (29). One of the oldest observations in acid-base physiology is that the urine becomes alkaline following a meal. The mechanism of this "alkaline tide" might involve, as Brodsky suggests, the secretion of some hormone such as VIP in response to ingestion of a meal.

C. Cell calcium and the calcium ionophore A23187

Ehrenspeck found that addition of the calcium ionophore A23187 stimulates electrogenic \( \text{HCO}_3^- \) secretion in a manner similar to the effect of cyclic AMP (31). Although this implies that cyclic AMP might increase intracellular calcium there is at present no evidence for it. Further, it is not sufficiently appreciated that the calcium ionophore is a neutral calcium:proton exchanger. Hence, its addition might lead to cell alkalinization. Despite these qualifications, the observation that electrogenic \( \text{HCO}_3^- \) secretion was stimulated is intriguing and does suggest that perhaps the apical chloride channel has been opened. Recent studies by Frizzell et al in tracheal mucosa have demonstrated that the apical chloride channel is sensitive to calcium. Obviously more work needs to be done in this area.
4. EPITHELIAL POLARITY OF PROTON AND BICARBONATE TRANSPORT

Epithelia are sheets of cells connected together by tight junctions and resting on a basement membrane. The plasma membrane of the epithelial cell is differentiated into two domains, apical and basolateral, which differ in composition and function. It is this difference in properties of the two domains that allows the epithelial sheet to perform its major function of transepithelial transport of solutes and water. Transport of these molecules is mediated by membrane proteins that are synthesized on ribosomes that are bound to the endoplasmic reticulum. The nascent proteins then traverse the golgi cisternae before they are carried to the plasma membrane by small vesicles. During this journey, a decision must be made regarding the ultimate destination of a membrane protein in an epithelium. The sorting of membrane proteins to apical or basolateral membrane remains one of the major problems in epithelial biology whose molecular mechanism that underlies this process remains obscure. Blobel had suggested that membrane proteins contain "topogenic" sequences that direct the protein to its ultimate destination. Using viruses whose budding is polarized investigators have mapped the pathway of the apical and basolateral proteins and identified the point at which they diverge. It appears that sorting occurs in a compartment, the trans golgi reticulum, that follows the most distal golgi cisterna. Further, apically directed proteins go directly to the apical membrane without first residing in the basolateral membrane. Similarly, basolateral proteins are sorted directly to the basolateral membrane (for review see 77).

Plasticity of Epithelial Polarity in the Intercalated Cell (73)

Secretion of protons or HCO₃⁻ in the intercalated cell is mediated by transport proteins that are deployed in the apical and basolateral membranes of the intercalated cell (Fig. 2). The finding that proton secretion into the urine frequently co-exists with bicarbonate secretion suggests that the two processes can be coordinately regulated to provide a wider repertoire of responses. In the rabbit, the cortical collecting tubule frequently secretes bicarbonate while the medullary tubule secretes acid into the urine. However, the number of intercalated cells in both is the same. Further, the capacity of the cortical collecting tubule and urinary bladder to secrete acid can be uncovered or increased by feeding the animal an acid diet. Recent studies have now documented that proton secretion and bicarbonate secretion are mediated by intercalated cells with reversed functional and structural polarity. Table 1 shows the differences between the two cell types. By using the same transport functions deployed on different membrane domains these cells exhibit reversed functional polarity; i.e. one secretes protons and the other bicarbonate. Some aspects of their structural polarity is also reversed. Endocytosis is vigorous in the apical membrane of the H⁺-secreting cell but is completely absent from the apical
membrane of the HCO₃ secreting cell. This characteristic allows these two cells to be distinguished in fixed sections by electron microscopy, Fig 5. Similarly, increasing the ambient pCO₂ causes apical exocytosis in the H⁺ secreting cell but basolateral exocytosis in the HCO₃ secreting cell. However, not all of the characteristics of the two cells are reversed. For instance, the HCO₃ secreting cell displays a peanut-lectin binding protein that is located on its apical membrane (54) while the H⁺ secreting cell has much less staining for this protein and frequently shows none. (In tubules perfused with peanut lectin before fixation, the H⁺ secreting cell endocytoses the peanut lectin which might cause some confusion). The cytoplasm of these two cell types also shows polarity in that the nucleus is located in the basal half of the cell and the tight junctions are near the luminal surface in both types of cells. Although both types of cells show subapical vesicles, the acid secreting cell demonstrates active fusion events with the apical plasma membrane, Fig.5, left panel. In the HCO₃ secreting cell, however, the subapical vesicles are separated from the apical plasma membrane by a dense mesh of cytoskeleton, Fig 5, right panel. Further, in this latter type of cell one can frequently demonstrate fusion events or omega figures in the basolateral membrane (Fig. 5 inset). Hence, when we say that there is reversed epithelial polarity in the two cell types, it should not be taken to mean that the two cells are symmetrically inverted; rather there are obvious and some subtle differences between the two cell types.

What is the basis for this specialization in structure and function in a cell type that is already a specialized cell among other specialized cells? There are at least two possible interpretations. We can think of the original intercalated cell as a parental cell line which produced two lineages that are now committed to their present terminally differentiated state. Alternatively, the intercalated cell is an epithelial cell whose properties are "plastic"; i.e. it will show structural and functional changes in response to environmental stimuli. There are probably other possible scenarios, but the above two can be experimentally distinguished. Recent studies have shown that the intercalated cell exhibits plasticity in epithelial polarity.

The cortical collecting tubule secretes HCO₃ into the lumen. (This may reflect the fact that rabbits eat an alkaline ash diet.) When rabbits are fed an acid diet for a few days the direction of HCO₃ movement is reversed to HCO₃ absorption, i.e. H⁺ secretion. Since the transport rates that are measured are net values in a tubule that contains both types of cells there were two possible interpretation of this finding; in one, the capacity of the H⁺ secreting cell to secrete protons is increased much greater than the amount of HCO₃ added to the lumen by the other cell type. In the other possibility, the HCO₃ secreting cell reversed its epithelial polarity to become a H⁺ secreting cell. To distinguish between these two possibilities we
counted the two cell types in control and in acid fed animals. The animals were fed NH₄Cl by stomach tube and it was found that although their plasma HCO₃ concentration decreased initially to low levels, this value recovered back to the original level within less than a day. Cortical collecting tubules were then dissected from control and acid-fed animals and the number of H⁺ secreting and HCO₃ secreting cells was measured. To count the number of H⁺ secreting cells we perfused the tubules with rhodamine labelled albumin. This is an excellent identifying marker since the other cells in the tubule (i.e. the principal cells and HCO₃ secreting intercalated cells) do not have active luminal endocytosis. After the cells internalized the albumin, the tubules were cooled down to low temperatures to reduce the rate of endocytosis and the tubule was perfused with fluorescein-linked peanut lectin. As can be seen in Table 1, these should provide a means for counting both cell types by the use of independent identifying labels. The results are shown in Fig 6. The total number of intercalated cells was the same in control and acid-fed rabbits, but the number of H⁺ secreting cells increased by a factor of ten, while the number of HCO₃ secreting cells decreased by the same number. These results are most compatible with conversion of epithelial polarity. However, they do not rule out the possibility that the H⁺ secreting cells proliferated while the HCO₃ secreting cells died and that the two processes occurred with sufficiently similar rates to account for a constant total number of intercalated cells. Since a ten fold increase in the number of H⁺ secreting cells within 1-4 days should have been accompanied by an increased appearance of mitotic figures, we assayed the frequency of mitotic figures in the collecting tubules and found no difference between control tubules and those removed from acid-treated animals. In fact there were no mitotic figures in any cell type in these tubules even though we would have predicted 5-10 /tubule. It is on the basis of these experiments that we concluded that there was plasticity in epithelial polarity.

The most direct method to demonstrate that there is actual conversion of polarity is to start with a pure population of cells and attempt to induce the change. Preliminary evidence in our laboratory has now demonstrated that in isolated HCO₃ secreting cells, at least the initial events in the conversion of epithelial polarity can be induced in vitro by reducing the intracellular pH of the cells (J. van Adelsberg, C. Cannon and Q. Al-Awqati, unpublished).

Other examples of Reversible Polarity

We can paraphrase these observations by concluding that epithelial polarity of the intercalated cell is not a fixed terminally differentiated state; rather, it is under physiological regulation. Other investigators have also been able to induce changes in the polarity of some epithelial
sheets. Mauchamps et al showed that when thyroid cells are grown at low serum concentration they form spheroidal epithelial structures with inwardly located apical surfaces and tight junctions (62). When the serum concentration is increased, the cells reverse their polarity so that the basolateral membranes now face the inside medium of the follicle which no longer contains colloid. Transitional forms of these cells can be seen and it appears that the first change is that tight junctions start to form at the other pole before intracellular organelle distribution and apical membrane specialization start their migration. A similar kind of experiment has been described by Stern in the chick epiblast (84). This is the first tissue to develop in the embryo that has clear structural and functional evidence of epithelial behavior. It can generate a reasonably high transepithelial potential difference that is due to the transport of sodium by amiloride-sensitive pathways. Like many other high-resistance epithelia, the orientation of the potential is lumen side negative. If the epiblast is mounted in organ culture and voltage-clamped to reverse the orientation of the membrane potential, there was a rapid reversal of a morphological marker of epithelial polarity, the presence of glycosaminoglycan granules in the basolateral pole of the cell. The Na, K, ATPase also shifted to the apical membrane although the results were not as complete as the granules within the short time (1-3 hrs) of the experiments. When the epithelium was voltage clamped to the reversed potential the transepithelial resistance immediately fell but recovered within 1-1.5 hrs to the original level suggesting that the tight junctions break early and then reform at the other pole of the cell. Similar studies to those have also been performed in sea urchin intestine. We would like to distinguish our findings from those described above. First, the changes we observed were limited to a single cell type rather than to all of the cells in the epithelial sheet. Second, the reversal of polarity was not complete. The tight junctions remained near the apical membrane and the nucleus remained near the basal surface. The major difference was a change in the composition of some membrane proteins and functions such as endocytosis and exocytosis.

Without limiting oneself to a parochial view of polarity, we must keep in mind that epithelial polarity is simply one example of polarization in other cells. For instance neurons exhibit clear polarity where the structure and function of the nerve terminal is quite different from that of the cell body. An interesting example of "plasticity" in polarity was demonstrated by studies in white cells which can intercalate and migrate across epithelial or endothelial sheets. It was found that when chemotactic peptides were added to one side of the epithelium white cells rapidly migrated towards that side and their chemotactic receptors moved to the "leading edge". This was reversed if the peptide was now washed from one side and added to the other (95).
Potential mechanisms for plasticity in intercalated cell polarity

When the animals were fed an acid diet, their plasma pH fell to low levels. This is the most likely initial signal. (Of course, at present, one cannot rule out a complex cascade whereby the acid diet leads to release of various hormones). Our preliminary in vitro studies, indeed, suggest that the initial events in the conversion of polarity are mediated by decreases in external and cytoplasmic pH. The most striking thing about the change in plasma pH is that it was transient. Further, this transient change led to a longterm event. The evidence that the event is longterm, comes from the observation that the epithelial polarity of the intercalated cell remains reversed after the tubules are taken from the acid-treated animal and bathed by artificial balanced salt solutions at pH of 7.4. Our recent in vitro studies also show that a transient cellular acidification can lead to longterm changes. The reason for emphasizing this characteristic is that it places certain restriction on the type of mechanism that can cause these changes.

When one discusses regulation of biological processes it is always in terms of reversible reactions. The most widely studied process is protein phosphorylation. Whereas kinases phosphorylate proteins to alter their function, there are also phosphatases that remove the covalently added phosphate group to return the protein to its "ground-state". Application of the signal should result in phosphorylation and removal of that signal would then result in dephosphorylation. In the case of the reversal of polarity, phosphorylation, in its simplest reversible form, cannot account for the observation. Recall that removal of the signal, i.e. return of the blood pH to its original level, does not result in return of the polarity to its original orientation. Hence, we have to search for a mechanism that can initiate a change which will not be reversible once the signal is removed. Such mechanisms must be thought of in the mediation of another, more familiar, process, which is memory. In that process, a short event can lead to a change that can be remembered for as long as the life of the individual. To restate the problem, a short acid-induced signal must cause a response that alters a protein(s) for long periods. If the change in the protein lasts for a few hours to days, i.e. it is of the same order of magnitude as the life of the protein, the mechanism to be searched for will involve changes in the rate of protein synthesis or degradation. If the induced change lasts for much longer, the mechanism must include some element of replication of the altered protein. Such mechanisms could include activation of cellular oncogenes. A recent interesting example has shown that a calcium activated protein kinase can phosphorylate itself, and that this autophosphorylation converts the enzyme into a calcium-insensitive form. This new calcium insensitive enzyme can
phosphorylate newly synthesized protein kinases thereby prolonging the effect of the phosphorylation. Clearly a detailed analysis of the time it takes to induce the changes in polarity, as well as the stability of these changes needs to be determined.

The conversion of the HCO₃ secreting cell to a H⁺ secreting cell requires that the apical Cl:HCO₃ exchanger be removed and the same or a different one inserted into the basolateral membrane. The apical peanut lectin binding protein would also have to be removed. Finally, the block on exocytosis and endocytosis of the sub-apical acid vesicles will have to be relieved to allow insertion of the proton ATPase into the apical membrane. There should also be coordinate inhibition of the basolateral fusion of acid vesicles. It is quite unlikely that a single mechanism can account for these diverse changes. An approach which we have recently taken is to identify early events in this response. We tested the effect of acidifying the cell on apical Cl:HCO₃ exchange in isolated HCO₃ secreting cells. We found that exposure of the cells to an acid external medium for only a transient period results in longterm inhibition of Cl:HCO₃ exchange. Further, we found that this effect is mediated by a change in intracellular calcium since removal of calcium prevents the effect of the cell acidification. An increase in cell calcium might activate a protease which will digest the Cl:HCO₃ exchanger; alternatively, calcium might activate a kinase which could phosphorylate and inhibit the transporter. A hypothesis we are testing is that the subapical vesicles are prevented from fusing with the apical membrane by a dense cytoskeletal band. Acidifying the cell by increasing cell calcium might lead to disaggregation of the cytoskeleton to relieve the block on fusion.

Implications for sorting of membrane proteins

Study of the plasticity of epithelial polarity of the intercalated cell promises to shed light on the mechanism of sorting of apical and basolateral membrane proteins. A number of potential mechanisms can be responsible for this conversion of epithelial polarity. The apical and basolateral forms of the Cl:HCO₃ exchangers might be fundamentally different proteins. The signal to the conversion of polarity might then involve coordinate induction of the synthesis of the appropriate pair of proteins as well as repression of the synthesis of the others. Alternatively, they may be the same proteins except that the signal to the change in polarity might induce a covalent modification that might act as a topogenic sequence for the appropriate membrane. A third mechanism is that organization of the cytoplasm might be responsible for determining the ultimate destination of the protein and that re-organizing the cytoskeleton might result in altering the desination of the protein.
6. DEVELOPMENTAL BIOLOGY OF THE INTERCALATED CELL

Renal tubular cells arise from the mesenchyme and develop in three "waves". The pronephros forms and atrophies. The mesonephros remains in the adult by being incorporated into the male genital system. The metanephros develops from the mesenchymal cells (the metanephric blastema) around the ureteric bud which arises from the lower end of the mesonephric (Wolffian) duct. Morphological studies have clearly documented the presence of intercalated cells in the mesonephric duct as well as in adult tissues originating from it such as the epididymis (14). Since the adult collecting tubules originate in the ureteric bud, it appears that the intercalated cells of the urogenital tract are derived from the mesonephric duct.

Each segment of the renal tubule is composed of cells that are homogenous in structure and function. The only exception is the cortical and medullary collecting tubules where within each segment there are two types of cells; the principal and intercalated cells. The minority cell, the intercalated cell, is usually surrounded by principal cells, although frequently one sees two intercalated cells side by side. The latter suggests that a cell has divided to generate a pair of cells. What is the origin of these cells? This is unknown at present but we speculate that they may have migrated there from somewhere else. Migration of cells during embryonic development has been well studied especially in the case of neural crest cells. Another potential source of migratory cells are tissue type macrophages. There are some similarities between intercalated cells and some tissue type macrophages such as osteoclasts. Both cells are enriched in carbonic anhydrase as exemplified by the genetic disease whose phenotype is osteopetrosis and renal tubular acidosis. Both seem to have band 3 analogues. When osteoclasts attach to bone, their intracellular vesicles polarize to face the bone and fuse with the membrane. They pump protons (and release lysosomal hydrolases) into the enclosed space which is the basis for their action in dissolution of bone. Macrophages can migrate and presumably intercalate between epithelial cells. However, before this idea can be taken seriously direct tests of the similarity between intercalated cells and bone marrow derived cells will need to be performed using surface markers or reconstitution of the bone marrow of irradiated animals with bone marrow from a genetically well defined donor.
TABLE 1
Polar Distribution of Structural and Functional Elements in the two types of Intercalated Cells

<table>
<thead>
<tr>
<th>Element</th>
<th>H⁺-SECRETING</th>
<th>HCO₃⁻-SECRETING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut Lectin binding</td>
<td>absent</td>
<td>apical</td>
</tr>
<tr>
<td>Cl⁻:HCO₃⁻ exchange</td>
<td>basolateral</td>
<td>apical</td>
</tr>
<tr>
<td>band 3 staining</td>
<td>apical</td>
<td>none</td>
</tr>
<tr>
<td>Proton ATPase</td>
<td>basolateral</td>
<td>basolateral</td>
</tr>
<tr>
<td>Acid vesicles</td>
<td>Sub-apical</td>
<td>Sub-apical and</td>
</tr>
<tr>
<td>endocytosis</td>
<td>apical</td>
<td>basolateral</td>
</tr>
<tr>
<td>CO₂-stimulated exocytosis</td>
<td>apical</td>
<td>basolateral</td>
</tr>
<tr>
<td>Cl⁻ Channel</td>
<td>? basolateral</td>
<td>? basolateral</td>
</tr>
<tr>
<td>Spectrin</td>
<td>basolateral</td>
<td>N.D.</td>
</tr>
<tr>
<td>Band 4.1</td>
<td>basolateral</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dense cytoskeleton</td>
<td>none</td>
<td>Sub-apical</td>
</tr>
<tr>
<td>Apical surface</td>
<td>microvilli</td>
<td>?microvilli</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Fig. 1. A model for transepithelial H⁺ secretion by the intercalated cell.

Fig. 2. Characteristics of the two types of intercalated cell, left shows HCO₃⁻-secreting cell while right panel shows the H⁺-secreting cell.

Fig 3. H⁺ transport and the development of a membrane potential by the proton translocating ATPase of golgi membranes (37). Proton transport (top panel) was measured as the uptake of a weak base (acridine orange) from the medium using a dual wavelength spectrophotometer. Note that addition of valinomycin, by collapsing a membrane potential accelerates the development of the pH gradient. Membrane potential (bottom panel) was measured as the ejection of the cationic dye, diS-C(3)-5. Addition of the neutral proton ionophore nigericin increases the apparent membrane potential, as does replacement of chloride by the impermeant gluconate.

Fig 4. Regulation of transepithelial H⁺ secretion in the turtle urinary bladder by CO₂. The first panel shows the effect on the short circuit current. The second shows the effect on secretion of fluorescent dextran which had previously been internalized into acid vesicles. The third panel shows the effect of CO₂ on intracellular clacium as measured by excitation ratio fluorometry using quin 2. The third panel shows the effect on cell pH as measured by the excitation ratio fluorometry of 5,6 carboxy fluorescein.

Fig 5. Electron micrographs of the two types of intercalated cell in rabbit cortical collecting tubule. The left panel shows the H⁺ secreting cell with typical fusion events on the apical membrane. Right shows the HCO₃⁻ secreting cell with its characteristic subapical cytoskeletal mesh; the inset shows basolateral fusion figures and subapical coated vesicles. L refers to the lumen.

Fig 6. Plasticity of epithelial polarity in the rabbit cortical collecting tubule. Bicarbonate and proton secreting cells were counted before and after treatment of the rabbits with an acid load. Bicarbonate secreting cells were identified by staining with fluorescein-labelled peanut lectin. The proton secreting cells were identified as those cells which endocytosed rhodamine labelled albumin.


References


