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Topical Review

Proton/Hydroxyl Transport in Gastric and Intestinal Epithelia

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Proton transport across the plasma membrane of the gastrointestinal epithelium occurs by various pathways. There is the permeability of H^+ across the lipid components of the membranes, probably of minor significance at physiological pH, but at the pH of the secretory surface of the parietal cell a factor that cannot be neglected. Transport of H^+ dependent on the protein components of the plasma membrane involves various mechanisms. For example $Na^+:H^+$ or $Cl^-:HCO_3^-$ antiport (exchange) are generally electroneutral mechanisms (i.e., neither affected by potential gradients nor affecting membrane conductance) that are widely distributed throughout the body. Plasma membranes may contain proton or bicarbonate conductances (i.e., gradients of either ion may be determined by the potential across the membrane). This type of pathway is often of minor significance, hence the electrical component of hydrogen ion gradients across the plasma membrane can often be neglected. In the case of the gastric parietal cell, proton transport depends on the activity of a specific ATPase. This ATPase may be present elsewhere in the intestinal tract.

This review will consider many of these proton pathways. In the case of brush border pathways, some of the data presented on $Na^+:H^+$ antiport will be derived from studies done on renal brush border rather than those of the small intestine, on the assumption that the properties of the antiporter are similar in the two tissues.

Lipid Permeation of H^+

The permeability of artificial liposomes to H^+ (or OH^-) is of the order of 10^{-4} cm sec^{-1} (Nichols & Deamer, 1980). This is comparable to water permeability and requires a specialized mechanism. One possibility is protons jumping along strands of water molecules extending into the bilayer. Electrical measurements of H^+ conductance indicate that the permeability by conductive pathways is about 10^{-9} cm sec^{-1}, orders of magnitude lower than the permeability measured by flux methods. This implies that H^+ transport across phospholipids is largely by electroneutral pathways.

Recent measurements of the characteristics of net HCl flux across planar bilayers showed that the calculated HCl permeability was very high (2.9 cm sec^{-1} for phosphatidylcholine) but only less than 1% could be accounted for by electrical pathways as for liposomes (Gutknecht & Walter, 1981). The flux was proportional to the HCl concentration and to the activity gradient. Hence, it was concluded that the transport was due to the electroneutral flux of HCl across the bilayer, perhaps in gaseous form.

It would appear unlikely that the structure of the lipid bilayer in natural membranes is such that these mechanisms of proton movement are absent. It is not possible to selectively remove the protein component without disturbing the lipid component in natural membranes. It is possible, however, to measure the leak of proton gradients in vesicles derived from the renal brush border, as shown in Fig. 1.

Here a pH gradient is imposed across the membrane by adding a sample of the vesicles pre-equilibrated at pH 5.5 to medium at a higher pH, 7.5 for example. The presence of a pH gradient is detected by the absorbance change of acridine orange caused by pH gradient-dependent uptake and stacking of this weak base. The decay of the change in optical density gives a measure of the stability of the pH gradient. In SO_4^{--}-containing solutions, the $t_{1/2}$ of decay is about 100 sec.

In the presence of 10 mM Cl^- this is significantly reduced. This can be interpreted as evidence for

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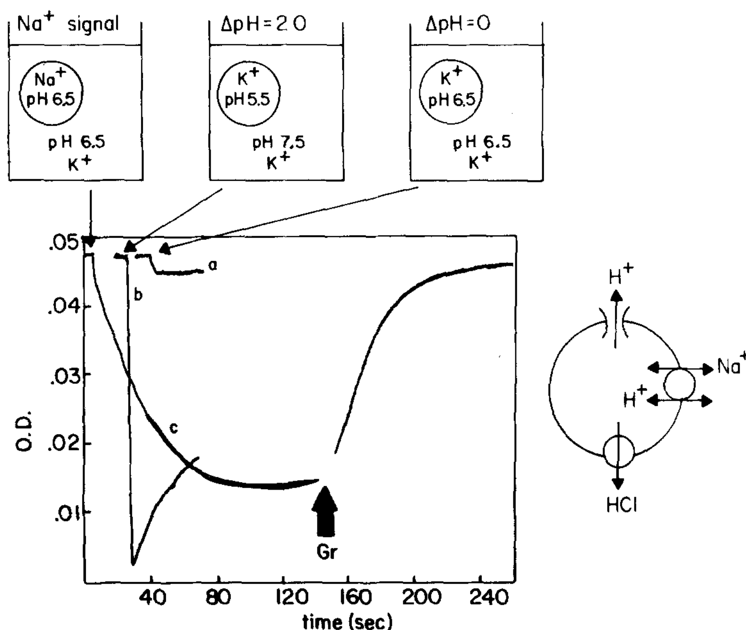

Fig. 1. Proton gradients in renal brush border vesicles. The diagrams at the top indicate the experimental conditions. The standard $Na^+:H^+$ exchange assay using the metachromatic shift of the acridine orange signal is Na^+ 180 mM intravesicular diluted into isotonic K_2SO_4 in the medium, and the optical response is shown in curve *c*. At the arrow where a steady-state pH gradient obtains, 1 µg/ml gramicidin dissipates the gradient. pH pulse calibration uses vesicles preincubated at pH 5.5 in K_2SO_4 and diluted into medium at pH 7.5, as shown in curve *b*. The $t_{1/2}$ of decay is 120 sec. Curve *a* shows that in the absence of a pH gradient there is no change of acridine orange absorbance

a leak of HCl across the renal brush border membrane. The addition of K^+ and valinomycin, a conductive K^+-selective ionophore also accelerates the rate of gradient dissipation, although not as much as the presence of Cl^-. This supports the idea that a proton conductance is present in the membrane. Alternatively, the addition of valinomycin to brush border vesicles with a K^+ gradient present across the membrane (in to out) results in acidification of the vesicle interior. This is due to coupling of the K^+ diffusion potential to H^+ flux through an H^+ conductance. Thus in the renal brush border, both HCl cotransport and H^+ conductance pathways are present (Burnham, Muenzesheimer, Rabon & Sachs, 1981); considering the relatively large contribution of HCl flux across lipid bilayers as compared to the conductive movement of H^+, and the relatively high H^+ conductance found in the renal brush border, it would appear possible that, whereas the HCl leak is lipid dependent, the H^+ conductance may depend on protein-dependent pathways.

The contribution of these leak pathways to H^+ transport across the membranes of the gastrointestinal epithelium is unknown at present. Certainly the presence of a large chemical gradient of H^+, as exists across the secretory canaliculus membrane of the parietal cell (lumen pH of about 0.8, cytosolic pH of about 7.7) would result in HCl back leak across the lipid phase of the membrane based on the calculated HCl permeability of bilayers (Gutknecht & Walter, 1981). No peculiarity of the lipid composition of this membrane has been noted (Saccomani et al., 1979) nor is the % contribution of amino sugars (resulting in positive charge screening) sufficient to lead to the assumption that HCl back leak will not occur. However, the presence of a proton pump will compensate for the back flux of hydrogen ion.

It would seem therefore that, apart from the specific pathways to be discussed below, natural membranes possess significant leak pathways for the hydrogen ion both in the form of HCl symport and H^+ conductance. The relative contribution of lipid and protein to these leaks is not known at present.

Na:H Antiport

General Properties

$H^+:Na^+$ countertransport occurs in a wide variety of membranes. For example, in halobacteria, electrogenic H^+ for Na^+ exchange accounts for much of the Na^+ gradient maintained by these organisms (Lanyi & MacDonald, 1976). Electrogenic $H^+:Na^+$ exchange has not been demonstrated across the plasma membrane of eukaryotes. Alkalophile bacteria which require $Na^+:H^+$ exchange for growth in high pH medium have been shown to lose this ability as a result of a mutation which results in a loss of the antiporter as well as Na^+:solute symport (Guffanti, Cohn, Kabach & Kralwicke, 1981). This may indicate a similarity of the Na^+ site for H^+ exchange and solute cotransport, at least in these organisms.

The occurrence of $Na^+:H^+$ exchange has been demonstrated in the intestine and kidney proximal

tubule (Murer, Hopfer & Kinne, 1976). The high paracellular conductance of the intact organ has prevented detailed studies of the possible mechanisms of this process in these tissues until the advent of intracellular pH measurements (Boron & Boulpaep, 1981). The use of isolated brush border-derived vesicles has therefore proved invaluable.

Two groups of methods are available for the study of H^+ transport, whether coupled to ion gradients or other energy sources. These depend on measurements of change of intravesicular or extravesicular pH. External pH can be measured electrometrically using a pH electrode, or by changes in absorbance of a dye such as bromcresol green. To measure changes in intravesicular pH, changes in fluorescence, or absorbance of dyes such as atebrin, 9-aminoacridine and acridine orange which accumulate as a function of pH gradients can be used (Rottenberg, 1979). Quinine accumulates in acid spaces and also shows a fluorescence shift as a function of pH (Lee & Forte, 1980). On occassion, radioactive weak bases such as methylamine, benzylamine, imidazole, or aminopyrine can be used as an alternative to optical probes to measure vesicular acidification. So far, relatively few weak acids have been used that show optical responses. A dye which shows a pH-dependent shift due to changes in the intravesicular pH is pyranin (Biegel & Gould, 1981). Loss of protons from an intravesicular space is usually assessed by trapping of radioactive weak acids such as DMO or salicylate. Alternatively, H^+ gradient-dependent movement of Na^+ has been demonstrated (Murer et al., 1976), where a pH gradient has been shown to drive uptake of $^{22}Na^+$.

Most studies on $Na^+:H^+$ exchange in brush border vesicles have been carried out by using inwardly directed Na^+ gradients. Acidification of the medium was measured by pH electrodes or alkalinization of the interior was assessed by DMO trapping (Kinsella & Aronson, 1980). The data we present here were obtained by using the uptake of acridine orange, as a convenient method of measuring changes of intravesicular pH. This technique is also more sparing of vesicles, in that the sensitivity of optical methods designed to measure intravesicular pH exceeds that of radioisotopic methods. This is useful where the quantity of material available may be limited, as in reconstitution studies.

In the absence of detailed studies in systems other than brush borders, we assume that the properties of the $Na^+:H^+$ antiporter described in what follows extends to other possible locations of this H^+ transport system.

Inwardly directed Na^+ gradients in brush border vesicles (these are oriented right side out) resulted in efflux of H^+ ions (Murer et al., 1976). Inasmuch as the rate of efflux was not affected by the addition of lipid permeable cations or anions it was concluded that the coupling between Na^+ and H^+ flux is by means of chemical rather than electrical gradients. At the same time that H^+ appeared in the medium, H^+ activity decreased in the vesicle interior as revealed by DMO uptake. Of the alkali metal cations, only Li^+ was able to substitute for Na^+ for the energization of H^+ movement (Kinsella & Aronson, 1980).

When outwardly directed Na^+ gradients were generated by preincubation of the brush border vesicles at Na^+ concentrations of 180 mM using sulfate as the anion, a 60-fold dilution resulted in a ΔpH of 1.6 units. Thus from a medium pH of 6.5 intravesicular pH fell to 4.9 (Fig. 1). The theoretical ΔpH is 1.8 for a 1:1 $Na^+:H^+$ exchange at a 60-fold dilution. The addition of gramicidin allowed the full ΔpH of 1.8 to develop by coupling Na^+ efflux to H^+ influx through the gramicidin channel. The shortfall in ΔpH due to the inherent $Na^+:H^+$ antiporter is therefore due to a balance between $Na^+:H^+$ exchange and proton gradient leak. The high activity of $Na^+:H^+$ exchange due to gramicidin obscures the leak component. As found earlier (Murer et al., 1976), the addition of a conductance pathway in the form of valinomycin in the presence of K^+ had only a small effect on the pH gradient due to the Na^+ gradient. This means that coupling between Na^+ efflux and proton influx is mainly chemical rather than electrical.

Measurement of the $K_{0.5}$ for Na^+ provided data that depended on the technique used. For outwardly directed Na^+ gradients, the K_m was about 80 mM for Na^+ and 7 mM for Li^+. If dissipation of preformed acid gradients by addition of Na^+ or Li^+ externally was used, the apparent K_m for Na^+ fell to 7 mM and that for Li^+ was unchanged. Measurements of changes of external pH showed that the apparent K_m for Na^+ was also in the low mM range (Kinsella & Aronson, 1980). This asymmetry of the affinity for Na^+ may indicate selective binding sites in the interior of the vesicle. There was a pH optimum of about 6.5 in terms of rate of gradient formation, and this suggested that a charged group was involved in the process of $Na^+:H^+$ exchange. In the case of a neutral channel, flux of H^+ would rise progressively with concentration and no pH optimum would be anticipated. Thus a protonatable group such as an imidazole or carboxyl group is likely to be involved in the rate limiting step for $Na^+:H^+$ exchange. Dicyclohexylcarbodimide, an hydrophobic carboxyl reagent, was without effect on $Na^+:H^+$ exchange, although ethoxycarbonyl ethoxy dihydroquinoline (EEDQ), a carboxyl activating reagent, did partially

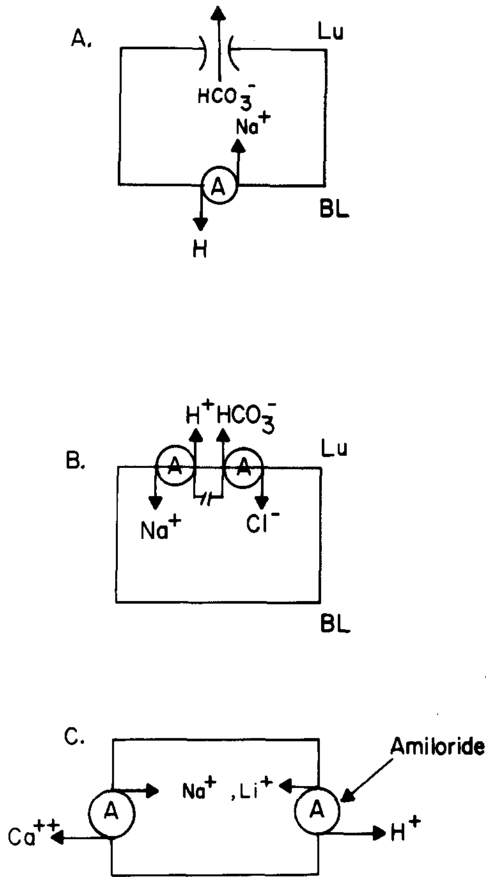

Fig. 2. Conceptual models of Na:H exchange in different cell types. (*A*): If Na^+:H^+ exchange is present at the basal-lateral surface, cell HCO_3^- will be at a higher activity than predicted from equilibrium distribution. If a HCO_3^- permeability is present apically, HCO_3^- secretion will result, dependent on the Na^+:K^+ ATPase maintenance of the Na^+ gradient. (*B*): If the Na^+:H^+ exchange is present at the luminal surface, acidification of the lumen will occur. If a HCO_3^- (OH^-):Cl^- exchange is also present, net NaCl uptake will occur with H_2O and CO_2 appearing in the lumen. With water accompanying salt entry, regulated volume increase of shrunken cells will result. (*C*): A model illustrating coupling between the Na^+, Li^+:H^+ exchange (Na^+:Li^+ exchange also occurs) and Na^+:Ca^{++} exchange, suggesting that hyperactivity of Na^+:H^+ exchange may result in elevated cell Ca^{++}

inhibit exchange. The nature of the ion binding site on the antiport protein is therefore unknown (Burnham et al., 1981).

The antiporter is stable to lyophilization, in contrast to many other Na^+-dependent systems in brush borders. It should be possible therefore to develop methods for reconstitution of this transport, perhaps with greater ease than has been the case for Na^+:glucose cotransport.

The presence of an HCl (or Cl^-:HCO_3^-) pathway in renal brush borders has been suggested since H^+ gradients were able to induce Cl^- movement (Warnock & Yee, 1981) across the membrane. Only part of this coupling was electrical. However, when measuring intravesicular pH changes using acridine orange, the change of pH due to inwardly directed Cl^- gradients was largely abolished by valinomycin $-K^+$. This was interpreted as showing that coupling between the Cl^- and H^+ gradients was mainly electrical in nature; i.e., the Cl^- diffusion potential determined H ion movement through an H^+ conductance.

Function of Na^+:H^+ Antiport

The presence of an electroneutral Na^+ for H^+ exchange in cells where Na^+ gradients are maintained by the Na^+–K^+ ATPase allows the mammalian cell to use this transport system for a variety of purposes. For example, as shown in Fig. 2, its presence on the basal surface of polarized epithelial cells could provide a means of increasing the intracellular HCO_3^- concentration. This may result in sodium pump-dependent alkalinization of the luminal surface as occurs in the stomach (Flemstrom, 1977). The presence of Na^+:H^+ exchange on the luminal surface results in both acidification of the lumen and Na^+ reabsorption. When coupled to HCO_3^-:Cl^- exchange, Na^+ entry by this means provides a mechanism for volume recovery of cells shrunk by exposure to a hypertonic medium. This process occurs by net entry of NaCl along with water.

An interesting facet of the antiporter is the possibility that it provides a leak pathway for Na^+ entry. Hyperactivity of this pathway will result, other systems being of normal activity, in higher intracellular Na^+ concentrations if Na^++K^+ ATPase does not compensate for Na^+ changes. In turn, if a major mechanism for Ca^{++} extrusion is dependent on Na^+:Ca^{++} exchange, then cell Ca^{++} will be set at a higher level. For cell processes such as smooth muscle contraction this will tend to hypersensitize the cells to a normal stimulus. It might be that the increased Na^+:Li^+ exchange seen in red cells of hypertensive patients reflects an increased Na^+:H^+ exchange capacity (Canessa et al., 1980). If a similar increase of antiport activity occurs in vascular smooth muscle, then the observed association between red cell cation exchange abnormality and hypertension may have a rational basis. Thus far, specific inhibitors of the antiport have not been discovered, but it may be anticipated that they would have wide therapeutic application. High concentrations (10^{-4} M) of amiloride inhibit Na^+:H^+ exchange (Kinsella & Aronson, 1980) and perhaps an analog of this drug may have higher affinity for the exchanger as opposed to the Na^+ channel. The drug inhibits Na^+:H^+ exchange in muscle also (Aicken & Thomas, 1977). The antihypertensive action of amiloride exceeds that expected from

its saluretic action, and the action in vascular smooth muscle may explain this clinical finding.

$Cl^-:OH^-$ or $Cl^-:HCO_3^-$ Exchange

The presence of $Cl^-:HCO_3^-$ exchange has been demonstrated in red cells and the responsible peptide has been isolated (Rothstein & Ramjeesingh, 1980). Valinomycin, which induces a K^+ conductance in the cell membranes, increased cation exchange in red cells but not net efflux unless an additional conductance was present such as proton conductance in the form of dinitrophenol (Pressman, 1976). Hence, HCO_3^-–Cl^- exchange is electroneutral. In gastric mucosa, there is absorption of CO_2 from the blood and secretion of HCO_3^- into the blood during acid secretion. The absence of any change in transmucosal potential due to HCO_3^- changes in serosal bathing solutions led to the conclusion that the $HCO_3^-:Cl^-$ exchange across the basal-lateral membrane was electroneutral (Rehm & Sanders, 1975). There is evidence for the presence of HCO_3^- conductance in other cell membranes. For example, in *Necturus* antrum, changes of HCO_3^- in the luminal solution have been reported to produce changes of potential difference (Flemstrom & Sachs, 1975).

In intestinal brush borders, evidence has been obtained for the presence of $Cl^-:OH^-$ exchange in addition to the $Na^+:H^+$ exchange discussed above (Liedtke & Hopfer, 1980). Combining the electroneutral Cl^- and Na^+ dependent exchanges, NaCl cotransport will result when NaCl gradients are present, due to coupled action of the antiporters. Thus to demonstrate directly coupled NaCl cotransport, it is not sufficient to show that there is Na^+ dependence of Cl^- flux or Cl^- dependence of Na^+ flux under gradient conditions. It is important to show Cl^- effects on $Na^+:Na^+$ exchange and *vice versa* under equilibrium conditions (Liedtke & Hopfer, 1980). Independent pathways for Na^+ and Cl^- would allow their individual regulation.

The existence of separate proteins for $Na^+:H^+$ and $HCO_3^-:Cl^-$ exchange allows for subtle regulation of cell pH and also the degree of coupling between Na^+ and Cl^- fluxes. Thus if the ratio of activities of these two antiporters is unity, no ΔpH will be driven by NaCl gradients and apparent cotransport of NaCl will be found. If the activity of the $Cl^-:HCO_3^-$ exchange is down regulated, then pH gradients will result from NaCl gradients and uncoupled Na^+ flux will be found. In renal brush borders, NaCl gradients equal to Na_2SO_4 gradients generate lower pH gradients (Burnham et al., 1981). The addition of gramicidin following dilution also produces much smaller pH gradients due to NaCl as opposed to Na_2SO_4 gradients. These data may be interpreted as reflecting a higher permeability of HCl as opposed to H_2SO_4 as well as a larger efflux of Na^+ as NaCl as compared to efflux of Na^+ by $Na^+:H^+$ exchange or Na_2SO_4 efflux. Warnock and Yee (1981) obtained evidence both for electrical and chemical gradient coupling of Cl^- and H^+ fluxes, and this would be consistent with the effects of the presence of Cl^- on the $Na^+:H^+$ antiporter-induced gradient.

HCO_3^- Secretion

HCO_3^- transport against electrochemical gradients has been demonstrated in the salivary duct (Knauf & Lubcke, 1975), gastric fundus (Flemstrom, 1977), antrum (Flemstrom & Sachs, 1975), and duodenum (Flemstrom, 1980). Although little is known about the mechanism, it appears that two types of secretion may be present. One is dependent on the presence of Cl^- in the lumen, and thus might be accounted for by $Cl^-:HCO_3^-$ exchange. The other is stimulated by prostaglandins and appears to be electrogenic (Flemstrom, 1980). Inhibition of the Na^+–K^+ ATPase inhibits the latter process, as it inhibits the secretion of HCO_3^- by the pancreatic ducts. In the latter type of HCO_3^- secretion, where coupling to Na^+ pump activity has been demonstrated, coupling may occur at the basal-lateral surface. For example, as shown in Fig. 2, the presence of $Na:H^+$ antiport on this surface would result in cellular HCO_3^- accumulation. If the apical surface is permeable to HCO_3^-, secondary active secretion of HCO_3 would occur. $K^+HCO_3^-$ cotransport could also occur, driven both by K^+ and HCO_3^- gradients.

An alternative mechanism that has been suggested for HCO_3^- secretion is based on the putative presence of an anion-sensitive Mg^{++} ATPase in, for example, renal brush borders (Murer, Kinne-Saffran, Beauwens & Kinne, 1980). Whereas there is agreement that a Mg^{++} ATPase of unknown function is present in these membranes, there is considerable dispute as to the origin of the ATPase found in many plasma membrane preparations. In our opinion there has as yet been no convincing demonstration of transport of H^+ or OH^- due to this ATPase. If HCO_3^- were pumped towards the cytosolic face of the pump, then the effect of the presence of this enzyme would be to acidify the luminal face. The mitochondrial ATPase, also present in the membrane of the chromaffin granules (Banks, 1965), would show such characteristics, including HCO_3^- activation and the same direction of acidification. Hence, it is difficult to exclude contamination by this mitochondrial pump in

membrane fractions as an explanation for the presence of anion-activated ATPase. Transport of H^+ towards the cytosolic face of the pump would result in alkalinization of the luminal face of the cell surface, i.e., HCO_3^- transport. To date we are not aware of any demonstration in intestinal or renal brush border vesicles of the functioning of such an ATP-dependent pump.

It has been shown, however (Murer et al., 1980), that ATP incorporated into brush border vesicles is hydrolysed, that hydrolysis is stimulated by the cholesterol requiring ionophore filipin, and that acidification of the extravesicular space is induced by protonophores during hydrolysis. Unfortunately, these studies were performed at pH 7.4, where the hydrolysis of ATP results in the production of scalar protons.

Gastric H^+ Transport

Several decades of investigation in intact animals and in chambered gastric epithelia have not explained the basis of the largest ion gradient (ΔpH ca. 7 units) known in mammalian biology. The apparent solution to the problem was obtained in two models, the isolated rabbit gastric gland (Berglindh, Helander & Obrink, 1976) and gastric parietal cell derived vesicles (Lee, Simpson & Scholes, 1974).

The energy source for acid secretion was determined by using a derivative of the gastric glands, permeabilized by either electric discharge (Berglindh, DiBona, Pace & Sachs, 1980) or by digitonin (Malinowska, Koelz & Sachs, 1981). In this model, ATP penetrated the limiting membrane of the cell, allowing design of experiments to establish the primary energy source for the acid pump. The problem as defined in intact tissues was to decide between redox-dependent and ATP-dependent transport of H^+. The large mitochondrial content of the parietal cell suggested a role either for mitochondrially generated ATP or a redox reaction. In shock-induced leaky gastric glands the addition of ATP restored the ability of the cells to secrete acid even if mitochondrial respiration was inhibited by CN^-, N_3^- or amytal (Berglindh et al., 1980). This information virtually excluded any direct contribution of a mitochondrial redox pump. However, it was still possible to postulate that a plasma membrane redox system was contributing to the acid gradient. Therefore glands were made permeable by digitonin and acid secretion inhibited by oligomycin and anoxia; ATP was still able to restore secretion (Fig. 3). These experiments argue that ATP is the primary energy source for mammalian gastric acid secretion, since redox reactions would be absent.

Intact tissue studies showed that K^+ was required for acid secretion (Harris, Frank & Edelman, 1958). Similar data were obtained in isolated rabbit gastric glands, in that acid secretion was abolished when K^+ was removed from the medium (Berglindh, 1978). However, measurement of intracellular K^+ under these conditions showed that it was still about 65 mM, a level normally adequate for secretion. However, Na^+ removal along with K^+ removal restored the ability of the gastric glands to accumulate acid, showing that cellular Na^+ could inhibit acid secretion. By treating gastric glands with ouabain and amphotericin B in media where Na^+ and K^+ were varied, it was possible to establish that cellular K^+ was inhibitory to acid secretion and that the $K_{0.5}$ for cell K^+ in nonstimulated tissue was about 18 mM. Stimulation reduced the $K_{0.5}$ for K^+ to about 12 mM (Koelz, Sachs & Berglindh, 1981). Intracellular Na^+ inhibited acid secretion.

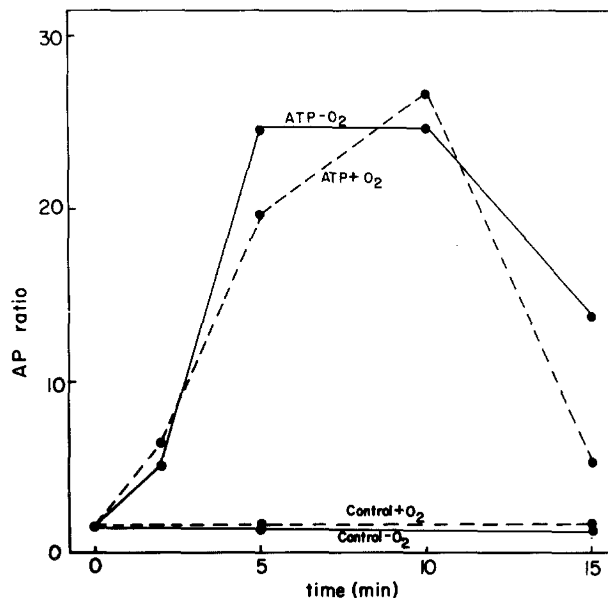

Fig. 3. An illustration of the ability of ATP to drive acid secretion in permeable gastric glands. The glands were inhibited with oligomycin, made permeable with digitonin, and ATP was added either in the presence or absence of oxygen (monitored by O_2 electrode). The same ratio of weak base (aminopyrine, AP) accumulation by the glands resulted

In permeable glands, when Na^+ was present in the medium, relatively high K^+ levels were found necessary for ATP restoration of acid secretion. However, when Na^+ was removed the apparent K_m for K^+ in terms of the ATP response in unstimulated glands was also reduced to 18 mM (Malinowska et al., 1981).

Thus in gastric glands the K^+ and ATP requirement for acid secretion could be established, as well as cellular Na^+ inhibition.

Gastric ATPase

The presence of a K^+ activated ATPase was demonstrated in frog gastric microsomes (Ganser & Forte, 1973*a*). It was also shown that K^+ active ionophores such as valinomycin activated the ATPase (Ganser & Forte, 1973*b*). This observation was followed by the finding that the gastric microsomes isolated from dog mucosa were able to accumulate H^+ when ATP was added to the medium in the presence of K^+ (Lee et al., 1974).

The transport reaction of the enzyme appears to be an electroneutral exchange of K^+ in the vesicle lumen for H^+ derived from the medium water (Sachs et al., 1976). A variety of experiments seems to establish this as a reasonable mechanism. Perhaps simplest is the observation that either preincubation with KCl or the addition of valinomycin is required for development of the pH gradient. This implies that K^+ has to penetrate into the vesicle lumen or occupy some intramembranal site for the exchange to occur. Loading vesicles with $^{86}Rb^+$ followed by addition of ATP results in the extrusion of $^{86}Rb^+$ by the ATPase (Schackmann, Schwartz, Saccomani & Sachs, 1977). As seen below, the effect of K^+ (or Rb^+) in accelerating ATPase activity or allowing H^+ transport is due to activation of hydrolysis of a phosphorylated form of the ATPase protein and K^+ must be present on the luminal face of the enzyme for activation of this reaction step. EEDQ inhibits the ATPase activity (Saccomani, Barcellona, Rabon & Sachs, 1980) but luminal K^+ uniquely protects against this inhibition. Thus the K^+ activating site is accessible only from the luminal face of the transport system.

The disappearance of H^+ from the medium was shown by alkalinization of the medium measured by pH electrodes or nonabsorbed pH-sensitive dyes. The appearance of protons in the vesicle lumen has been established by the accumulation of weak bases such as acridine orange, 9-aminoacridine, or aminopyrine (Rabon, Chang & Sachs, 1978) and by changes in fluorescence emission of quinine (Lee & Forte, 1980).

Comparison of the disappearance of medium H^+ and appearance of H^+ in the vesicle has led to the conclusion that, for example, whereas 50 nmol protons disappeared per µl vesicle from the medium at maximal gradient conditions. only about 1 nmol appeared in the vesicle interion based on the measured ΔpH of 3 units. Stated alternatively, the predicted internal pH, based on H^+ disappearance was about 1.7 with medium pH set at 6.1, whereas the measured internal pH was about 3. This discrepancy may be accounted for by buffering of the transported H^+ (Rabon et al., 1978). On the other hand, buffering does not account for the shortfall in the pH gradient as compared to the capacity of the putative gastric proton pump. One question is the fraction of the isolated vesicles actually transporting H^+ or K^+ following ATP addition. It might be anticipated that those vesicles which transport K^+ would be able to reduce the K^+ level from 75 mM to values of about 10 mM. A final intravesicular level of about $^1/_3$ the initial level is found from $^{86}Rb^+$ efflux experiments. This result would be expected if only two thirds of the vesicles as isolated were able to catalyze transport efficiently. This would increase the calculated ΔpH of the active fraction but would still be considerably short of the predicted ΔpH.

In relation to the pH gradient achieved by the vesicles, the stoichiometry of proton transport in relation to ATP breakdown is of interest. Here there is some disagreement between different laboratories. The stoichiometry at a medium pH of 6.1 appears in one laboratory to be about 1 or 1.5 depending on whether one considers total or only K^+ activated ATPase (Reenstra, Lee & Forte, 1980). In our laboratory using only K^+ activated ATPase for the calculation in a somewhat more purified preparation, over a range of ATP concentrations and either with no initial K^+ gradient or with an initial outwardly oriented gradient, the stoichiometry of H^+ transported per ATP hydrolyzed was found to be 2 (Sachs et al., 1980). In terms of H^+ appearance in the vesicle interior, the stoichiometry was considerably less. If only a fraction of the ATP hydrolysis results in vectorial H^+ transport, as discussed, then the stoichiometry has to be corrected by the nonproductive ATPase reaction and would be greater than 2. The large pH gradient in permeable gastric glands dependent upon an ATPase mechanism argues that the final stoichiometry cannot exceed 1. The problem of the ATPase/proton transport stoichiometry then reduces to the question of whether the stoichiometry is fixed at 1 and there are measurement artifacts in vesicles, or whether it varies as a function of the pH gradient or the preparation. A more remote possibility is very high phosphorylation potential at the secretory site, so that the ΔG_{ATP} is high enough to support a stoichiometry of 2.

The rheogenicity of H^+ transport has long been a major point of exploration in intact frog mucosa. Observations interpreted as indicative of rheogenicity are the fall in resistance associated with stimulation of secretion, and the identity between short-circuit current and H^+ ion transport rate in the absence of Cl^- with SO_4^{2-} as the substituting anion (Rehm, 1965). Both observations are compatible with a reheogenic H^+ pump mechanism but can also be explained

by an electroneutral mechanism. If the data are interpreted in favor of an electroneutral H^+ pump mechanism, the decrease of resistance can be explained by changes in the geometry of the secretory surface. Morphological changes observed with onset of secretion involved expansion of the surface area of the apical surface of the acid secretory cell. However, under certain conditions such as SCN^- inhibition it is possible to dissociate resistance changes and membrane area, at least in piglet mucosa (Black, Forte & Forte, 1980). Resistance changes may also be due to parallel pathways of ion permeability necessary for functioning of the transport ATPase. The effect of SO_4^{2-} substitution for Cl^- in the frog is not only to reverse transepithelial potential during acid secretion, i.e., lumen positive, but also to increase the K^+ conductance of the luminal face of the epithelium. The presence of a pump which maintains a luminally directed K^+ gradient across the apical membrane which is K^+ conductive would also result in a relationship between H^+ transport and short-circuit current as described above (Sachs et al., 1976). Thus the interpretation of electrical data obtained in intact mucosa is equivocal.

Electrogenicity of the transport reaction in gastric vesicles can be determined by the use of probes of vesicle potential during transport. In the case of a rheogenic H^+ pump, the vesicle interior would be expected to be positive with respect to the medium and hence accumulate lipid permeable anions, such as SCN^-. SCN^- accumulation was found only in the presence of valinomycin, following ATP addition to KCl-equilibrated vesicles. This was interpreted as indicating that the pump was not electrogenic in the H^+ transport direction, but that SCN^- was a competent probe of vesicle potential and was measuring the K^+ gradient by responding to the valinomycin-dependent K^+ diffusion potentials, the K^+ gradient being set up by the pump (Sachs et al., 1976). An electrogenic proton pump would also be unable to maintain a H^+ gradient in the presence of protonophores such as tetrachlorosalicylanilide (TCS). Although TCS reduced H^+ accumulation slightly, the major fraction of the H^+ gradient was maintained. These two pieces of information argue strongly against pump electrogenicity in the direction expected from intact tissue studies. In the vesicle preparation, the use of SO_4^{2-} rather than Cl^- also does not result in a potential difference due to ATP. However, piglet mucosa does not secrete acid in sulfate solutions (Forte & Machen, 1975), and it is not known whether a K^+ conductance is revealed in mammalian tissue by SO_4^{2-} substitution. Without lengthy preincubation it is also not possible to demonstrate proton transport in hog gastric vesicles in SO_4^{2-} solutions, and in rabbit

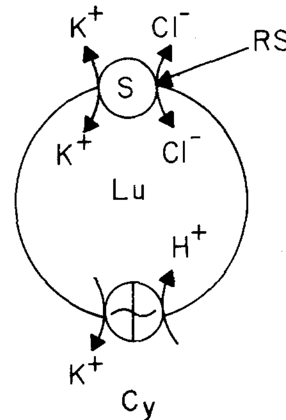

Fig. 4. A model of H^+ transport by vesicles isolated from the membrane of hog parietal cells. The pump (⊕) exchanges H^+ for K^+. K^+ entry into the lumen is indicated by a coupled transport process, the regulated site (*RS*), consisting of a K^+ path and a Cl^- path, each individually able to exchange K^+ for K^+ and Cl^- for Cl^- and, when coupled, to effect influx of KCl into the lumen of the secretory canaliculus

gastric glands SO_4^{2-} substitution for Cl^- abolished the ability to secrete acid. The morphological appearance of the parietal cell is distinctly abnormal in these solutions, and this may account for the difficulty of demonstrating acid secretion (H.F. Helander and T. Berglindh *unpublished observations*) in mammalian preparations with SO_4^{2-} substitution.

If the ATPase were electrogenic in the K^+ transport direction, the vesicle interior negativity would be detected by accumulation of lipid-permeable cations such as carbocyanine dyes. Again such accumulation was only detected in the presence of agents that modify conductance, in this case, protonophores, which allowed the membrane potential to approximate the H^+ ion diffusion potential (Rabon et al., 1978). Attempts to measure the stoichiometry of K^+ transport suggested that an equal number of H and K ions were transported by the pump and no net flux of Cl^- was detected from an equilibrium situation (Schackmann et al., 1977). Since there is no known way of short circuiting vesicle potentials without distorting membrane conductance, the equivalence of cation translocation follows from electroneutrality. However, the data from lipid-permeable ion probes of vesicle potential suggest electroneutrality of transport, and the failure of such ions to affect the rate of the transport reaction also argues for electroneutrality of transport.

The biological advantage of electrogenicity of the gastric proton pump is also questionable. Thus in the case of bacterial or mitochondrial proton pumps, or in the case of the mammalian Na^+–K^+ ATPase, the electrochemical gradient of H^+ or Na^+ is coupled to transport of other solutes. The rapid electrical re-

sponse of biomembranes compared to the slower development of chemical concentration gradients allows a constancy of the $\Delta\bar{\mu}$ to be maintained. There is no known transport function of the ΔpH generated by the gastric proton pump, hence no advantage in terms of electrogenicity is to be expected.

In summary, the vesicle studies performed suggest that the gastric ATPase exchanges H^+ for K^+, and the stoichiometry of cation per ATP lies between 2 and 1. The exchange also appears to be electroneutral within the limits of detectability of vesicle potential or effects of ionophores. A model for H^+ transport by gastric vesicles is shown in Fig. 4.

Reaction Cycle of the ATPase

The gastric ATPase, like the Na^+–K^+ and Ca^{++} ATPases, forms a phosphorylated intermediate. The reaction steps have been partially defined by both steady state and transient kinetic methods (Wallmark & Mardh, 1979; Wallmark et al., 1980; Stewart, Wallmark & Sachs, 1981). The salient observations are: (i) that there appear to be two ATP affinities, (ii) that K^+ stimulates ATPase activity at low and inhibits at high K^+/ATP ratios, but dephosphorylation is always stimulated by K^+, and (iii) that low cytosolic pH favors phosphorylation whereas high luminal pH favors cation-induced or spontaneous dephosphorylation.

A reaction cycle which partially explains these observations is given in Fig. 5. The enzyme reaction is initiated by binding of ATP to enzyme. Whether tightly bound divalent metal cation is required for this binding is not known, but added divalent cation is required for subsequent phosphate transfer from ATP to protein. There is competition between ATP and K^+ on the cytosolic face in terms of ATP binding and at high enough K^+ to ATP ratios that ATP binding reaction can become rate limiting for the overall ATPase activity. Decreasing the pH at the cytosolic face of the enzyme also facilitates K^+ dissociation. Hence the K^+ bound at the cytosolic face explains the inhibition of ATPase activity by high K^+ concentrations, and the H^+ requirement for phosphorylation correlates with the transport reaction of the enzyme.

Following formation of phosphoenzyme, there is loss of ADP to form the protonated EP form of the enzyme. Reversal of these reactions accounts for the ATP–ADP exchange activity of the enzyme. Translocation of the proton results in the formation of the ADP-insensitive form of EP, and the presence of low concentrations of K^+ on the luminal face of the enzyme results in rapid dephosphorylation to regenerate the $E:K_o$ form of the enzyme. This reaction

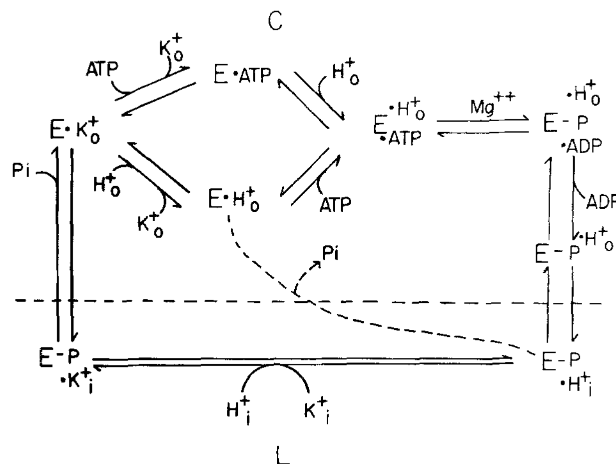

Fig. 5. A partial reaction sequence for the gastric H^+:K^+ ATPase. The details are discussed on the text and in Stewart et al., 1981. *C* represents the cytosolic side of the membrane barrier, and *L* the luminal face

cycle is similar in most respects to the reaction cycle of the Na^+K^+ ATPase. It should be pointed out that these data are obtained from half-cycle transient kinetics, and a more complex pathway probably obtains.

The correlation between the catalytic sequence and transport measurements is reasonable. For example, the requirement for K^+ in the vesicle interior for H^+ transport is explained by the K^+ requirement at this location for dephosphorylation. K^+ binding to the cytosolic face of the enzyme inhibits phosphorylation and results in inhibition of transport as well. Na^+ turns out to be an inhibitory ligand at this site as well, providing a partial explanation for the Na^+ inhibition of acid secretion in gastric glands mentioned above (Koelz et al., 1981).

The rate limiting steps depend on the conditions of study. In tight vesicles the entry of K^+ is limiting for the ATPase reaction. At high medium pH or high K^+ to ATP ratios the binding of ATP is rate limiting. At low lumen pH the rate of dephosphorylation may become rate limiting. Under optimal conditions, namely, neutral pH at both surfaces, low K^+ and high ATP levels at the cytosolic face, interaction between enzyme subunits may slow the overall rate.

Structure of the ATPase

Purification of the enzyme still in membrane form showed that 90% of the peptide components have a relative molecular weight of about 100,000 on gel electrophoresis (Saccomani et al., 1977). Studies on the sensitivity of the enzyme to trypsin showed that, in the absence of ATP, digestion for 30 min destroyed $^2/_3$ of the protein leaving a heavily glycosylated pro-

tein at the 100,000 mol wt region of the SDS gel. In the presence of ATP, enzyme activity was partially protected against tryptic inactivation, but still $^{1}/_{3}$ of the peptides were hydrolyzed (Saccomani et al., 1979*b*). These data indicate that the 100,000 mol wt region of the SDS-PAGE is heterogeneous. Further evidence for the heterogeneity of the protein is deduced from separation in isoelectric focussing gels, where a glycoprotein is found at an isoelectric point of 8.9 and a major group of glycoproteins is found between an isoletic point of 6.2 and 6.8, and a minor group has an isoelectric point of about 7.4 (A.J.M. Smolka, *unpublished*).

Electron irradiation at −40 °C shows that the effective molecular weight of the enzyme is about 300,000, indicating at least a trimeric structure. It is not known at present whether there are three distinct peptides, or, for example, two catalytic and one glycoprotein subunit (Saccomani et al., 1981). From phosphorylation data where at most 2 nmol P_i are incorporated per mg enzyme (less than 1 equivalent per mol) it would appear that non-ATPase protein must be present in the preparations. The role of the other peptides, such as glycoprotein, is not known.

The use of site-selective reagents has defined some amino acid residues involved at the catalytic center of the enzyme. For example, amino group reagents show the necessity of such residues for catalytic activity (Lee, Breitbart, Berman & Forte, 1979; Schrijen, Luyben, DePont & Bonting, 1980). SH reagents provide evidence for SH groups both at the catalytic center and elsewhere in the enzyme perhaps related to the transport reaction (Schrijen, Van Groningen-Luyben, DePont & Bonting, 1981). Histidine reagents and measurements of the relationship between K_m and pH indicate that a histidine residue is also required for ATP hydrolysis (Saccomani et al., 1981 *a*). The carboxyl activating reagent, EEDQ, inhibits the ATPase reaction, but its effect, as discussed above, is prevented by K^+ present at the luminal face, suggesting that cation binding at a luminally accessible K^+ site modifies the availability of an essential carboxyl group involved in the K^+ activation pathway (Saccomani et al., 1980). Evidently, these are only fragments of the structural information that will eventually be placed in a logical framework when the enzyme structure has been analyzed.

Regulation of the ATPase

Gastric acid secretion is a facultative process. The second messenger for histamine stimulation is cAMP (Harris & Alonso, 1965). Based on the properties of the gastric mucosa and the ATPase system, several possible sites of regulation can be considered. Substrate mobilization with access of reducing equivalents to the mitochondrial respiratory chain is a step necessary for acid secretion to occur (Hersey, 1974). Exactly at which point of metabolism this occurs is not known. The morphological rearrangement that occurs within the parietal cell is another possible site of action of second messengers (Sedar, 1965). Although it seems that output of acid into the gastric lumen does not occur without this morphological transformation, the transformation can be present without measurable acid secretion (Forte & Forte, 1975; Black et al., 1980). In isolated gastric glands in the absence of secretagogue there is still weak base accumulation, which increases virtually to stimulated levels with increasing medium K^+ (Koelz et al., 1981). Morphological correlates of this phenomenon are absent. Cytoskeletal elements such as microfilaments and microtubules are involved and their function may be regulated by cAMP and intracellular Ca levels (Kasbekar, 1978).

The major rate-limiting step in ATP hydrolysis by isolated vesicles from the microsomal fraction of most species studied is the entry of KCl. The entry is probably an electroneutral symport process (Malinowska et al., 1981; Sachs et al., 1976). It has been suggested that Ca^{++} (about 10^{-6} M) increases KCl permeability of gastric vesicles (Michelangeli, 1980), and recently a vesicle fraction has been isolated from secreting rabbit mucosa with a much higher KCl permeability than heretofore reported (Wolosin & Forte, 1981). It may be of more than passing interest to note that gastric vesicles show, in spite of a low net uptake of KCl (Rabon, Takeguchi & Sachs, 1980) or RbCl, a rapid cation-cation exchange, with a rate constant about 150 times that of the net flux (Schackmann et al., 1977). If this observation reflects a cation pathway distinct from the catalytic subunit, it implies that a Cl^-–Cl^- exchange path of equivalent potency must be absent, otherwise rapid net flux of KCl would result. Indeed Cl^-–Cl^- exchange is not found. Aging of the vesicles or mild tryptic digestion revealed a Cl pathway (Rabon, Kajdos & Sachs, 1979). We may speculate therefore that regulation of net KCl flux into the secretory lumen depends on activation of the Cl pathway to accompany the ever present K^+ pathway. Both components appear to be electroneutral, and in the intact cell the two fluxes appear absolutely interdependent. The rapid Cl exchange noted in intact frog mucosa may well reflect this postulated regulated exchanger. Other processes that also could be regulated are the removal of the $E.K_0$ form of the enzyme, the cytosolic pH, the level of ATP available to the enzyme, and the interconversion of the two forms of phosphoenzyme. The two most probable

points of regulation are the access of the enzyme to the lumen and the flux of KCl into the lumen of the secretory canaliculus.

Catalysis-Transport Coupling

Above, the transport reaction characteristics and the catalytic sequence of the gastric ATPase have been outlined. The relationship between these two sets of phenomena is virtually unexplored in the gastric enzyme, but its general similarity to the Na^+–K^+ ATPase makes some speculation possible.

Based on radiation inactivation observations, it appeared that the enzyme is a trimer of the 100,000-dalton peptides comprising it. Vanadate inhibits the enzyme at two sites, one of high affinity ($K_I = 12$ nM) and one of lower affinity ($K_I = 3$ μM). Inhibition of the high affinity site reduced activity by close to 50% (Faller et al., 1981). There are two ATP sites, of high and low affinity, with respect to the catalytic activity (Wallmark et al., 1980). Whereas EEDQ inhibition of the ATPase was prevented by luminal K^+, inhibition by EEDQ of the partial reaction, *p*-nitrophenyl phosphatase, is prevented to the extent of 50% by luminal K^+ and 50% by cytosolic K^+.

Data of this nature suggest that the gastric H^+–K^+ ATPase functions not as a monomer, but as a dimer, of the catalytic subunit. However, the mechanistic advantage of dimer function is not clear. The suggestion has been made, in the case of the Na^+–K^+ ATPase, that not only is there half-site reactivity, but that in the catalytic cycle, each half of the dimer is out of phase with the other (Repke & Schon, 1973). Studies of red cell and other Na^+–K^+ ATPases have suggested that Na^+ and K^+ induce different conformations of the enzyme, defined as the Na^+ form and the K^+ form (Post, Hegyvery & Kume, 1972). These have been demonstrated by fluorescence measurements with substrates such as FTP and with enzyme labeled with fluorescein (Karlish, 1980) as well as by the ionic requirements for pump reversal. Na^+ and K^+ apparently compete at the same sites, and it has also been shown that Na^+ and K^+ sites coexist on the enzyme (Sachs, 1977). This appears to suggest that the functional enzyme is at least a dimer. In the above, two sites may also be equated to two states of the enzyme.

Based on a likelihood that transport enzymes are transmembrane peptides, the intra-membrane segment is probably channel-like in structure with, as for the model peptide gramicidin (Eisenman, Hagglund, Sandblom & Enos, 1981), several ion binding sites (Urry et al., 1980). If it is supposed that an hydrophobically located carboxyl group binds luminal K^+, in the case of H^+–K^+ ATPase, the transport site, in contrast to gramicidin, contains charged groups.

An active transport reaction should involve both changes of barrier (gate) location and changes of affinity. With the Na^+–K^+ ATPase, it appears that occupancy of one Na^+ site suffices for the formation of *E-P*, but three Na^+ sites must be occupied before Na^+ transport can occur (R. Blostein, *personal communication*). The reaction is virtually specific for Na^+, except in the case where the dimer structure is disrupted by thiomerosal, an SH reagent, where Na^+ ATPase activity becomes Na^+ independent (Kaplan & Mone, 1981). The K^+ side of the reaction is less specific, even Na^+ being a weak ligand. In the case of the H^+–K^+ ATPase, it must be assumed that phosphorylation is proton specific, and for dephosphorylation as for the Na^+–K^+ ATPase, Na^+ can substitute for K^+, in that high concentrations of Na^+ stimulate dephosphorylation and Na^+ and K^+ can interact at the low affinity cytosolic face. The interaction of H^+ and K^+, as revealed by the antagonistic effects of these ions on formation and breakdown of *E-P* at either face of the enzyme, can also be interpreted as due to similar sites for these cations.

A hypothetical model for the gastric ATPase is shown in Fig. 6. The enzyme is modelled as a dimer, with alternate site phosphorylation and two gating mechanisms which are present in the nonmembranous segment (the major portion) of the peptide. Three states of the enzyme are illustrated. State *A* contains a monomer where the channel entry barrier is closed, but H^+ can bind to the transport site and ATP to the catalytic site. The other monomer contains bound K^+, the low energy form of *E-P*, and the cytosolic barrier is closed. State *B* has both barriers closed, and only one subunit is phosphorylated, monomer *2* having discharged P_i. This would correspond to occluded forms of $E \cdot K^+$ and $E \cdot H^+$. The *E-P* form on monomer *1* is high energy and available for ATP–ADP exchange. In state *C*, monomer *1* is still phosphorylated but the luminal barrier has decayed, resulting in ADP insensitive *E-P*. Monomer *2* has bound ATP and the cytosolic barrier for K^+ has decayed. With loss of K^+ and binding of H^+ monomer *2* converts to monomer *1* of state *A* and with loss of H^+ and binding of K^+ monomer *1* converts to monomer *2* of state *A*. The scheme then implies that the dimer exists in three states, containing six forms of the monomer.

The rapid kinetic results indicate that the reaction sequence *A1, B1* through *C1* occurs very rapidly in that ADP-insensitive *E-P* is the major form of *E-P* found after phosphorylation. Equally, the series of reactions *A2, B2* to *C2* also occur with a rate considerably greater than the overall velocity. At low luminal

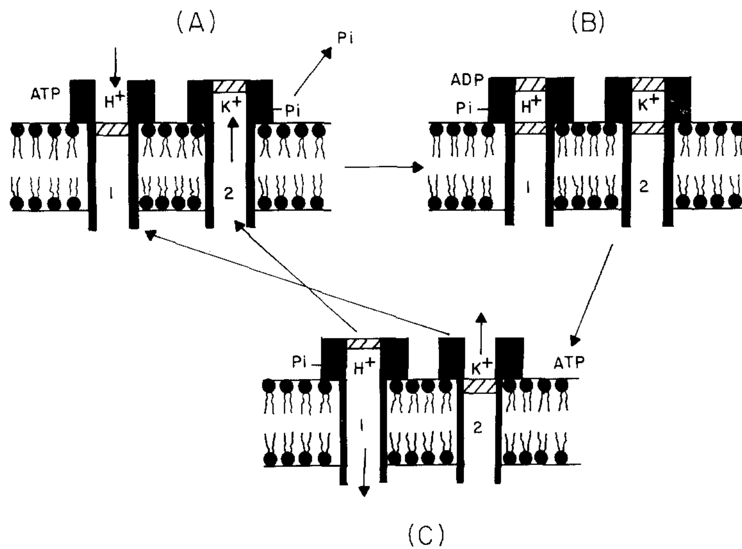

Fig. 6. An illustration of a dimeric pump mechanism. (*A*): Monomer *1* is protonated and binds ATP at a high affinity site, whereas monomer *2* has bound K^+ on the luminal face, and is in the E_2-P form. (*B*): Monomer *1* is phosphorylated, and E_1-P·ADP is formed with occlusion of H^+, and monomer *2* has lost P_i along with occlusion of K^+. (*C*): Monomer *1* loses ADP and releases H^+ into the lumen. K^+ binding interconverts monomer *1* to monomer *2* in (*A*). ATP binds to the occluded K^+ form at a low affinity site, with release of K^+ into cytoplasm. Binding of H^+ converts monomer *2* to *1*

pH, K^+-dependent dephosphorylation is slow, and this may be rate limiting for net ATPase reaction. Alternatively, maintenance of the need for dimer interaction may determine the overall reaction rate, transient kinetics being unable to determine the effects of dimer interactions.

Role of H^+–K^+ ATPase

Although definite proof that this enzyme occurs elsewhere in the body is absent, preliminary evidence using staining by monoclonal antibodies suggest that renal distal tubule and colon may contain structures similar to at least part of the ATPase (A.J.M. Smolka and G. Sachs, *unpublished observations*). It may be that this enzyme can act as a means of K^+ reabsorption or acidification in these tissues. Such an action of the enzyme has been detected in rabbit gastric glands suspended in Na^+-free ouabain containing solutions, in that K^+ accumulation under these conditions may be due to activity of the proton pump. Enzymes with somewhat similar characteristics occur in plant tissues (Sze, 1980), bacteria (Epstein, Whitelaw & Hesse, 1978), and perhaps yeast (Scarborough, 1976). Thus, although the gastric ATPase is a recently discovered eukaryotic enzyme, it or its subunits may have a wider distribution than heretofore recognised.

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