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The Chloride Pump: A Cl^--Translocating P-Type ATPase

George A. Gerencser
Department of Physiology, College of Medicine, University of Florida, Gainesville, FL 32610-0274

Referee: Dr. Chiyoko Inagaki, Dept. of Pharmacology, Kansai Medical Univ., Osaka, Japan

Address correspondence to: Dr. George A. Gerencser, Department of Physiology, College of Medicine, University of Florida, Box 100274, Gainesville, FL 32610-0274

ABSTRACT: Three widely documented mechanisms of chloride transport across plasma membranes are anion-coupled antiport, sodium-coupled symport, and an electrochemical coupling process. No direct genetic evidence has yet been provided for primary active chloride transport despite numerous reports of cellular Cl^--stimulated adenosine triphosphate (ATP)ases coexisting in the same tissue with uphill chloride transport that could not be accounted for by the three common chloride transport processes. Cl^--stimulated ATPases are a common property of practically all biological cells, with the major location being of mitochondrial origin. It also appears that plasma membranes are sites of Cl^--stimulated ATPase activity. Recent studies of Cl^--stimulated ATPase activity and chloride transport in the same membrane system, including liposomes, suggest a mediation by the ATPase in net movement of chloride up its electrochemical gradient across plasma membranes. Further studies, especially from a molecular biological perspective, are required to confirm a direct transport role to plasma membrane-localized Cl^--stimulated ATPases.

KEY WORDS: chloride pump, P-type ATPase, Cl^- transport.

I. INTRODUCTION

The electrical activity of isolated tissue has been a source of intense interest and much scientific study since the early reports of DuBois-Reymond[37] and Galeotti.[51] However, it was not until the brilliant and creative studies of Ussing[171] on isolated frog skin and, later, those of Leaf[123] and his coworkers on isolated toad urinary bladder that defined the nature of the bioelectric potential. The defined interrelationship between bioelectric potential and active Na^+ transport ushered in the modern era of ion transport study in epithelia and other tissues. Skou[161] molecularly defined the nature of Na^+ transport with his discovery of the (Na^+ + K^+)-stimulated ATPase enzyme. For years thereafter, active Na^+ transport across epithelia had occupied the collective focus of transport physiologists, with Cl^- assuming a secondary role of passive counterion. However, within the past 30 years there has been an intensive interest in transmembrane Cl^- transport, primarily because Cl^- has been found to move actively in a very wide range of species.[49,57]

In the last 30 years or so, three general mechanisms of transepithelial or transmembrane Cl^- transport have been reasonably well established. The first of these is a strictly

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passive means of Cl^- transport coupled electrically and/or chemically to primary active Na^+ transport, and is exemplified in isolated frog skin[170] and toad urinary bladder.[123] The second well-accepted Cl^- transport process is secondarily active and is thought to be effected through an electrically neutral Na^+-coupled carrier mechanism that drives Cl^- uphill into cells via the inward flow of Na^+ down a favorable electrochemical potential gradient. This NaCl cotransport process is located within the mucosal membrane if Cl^- is absorbed actively by the epithelium or is located within the basolateral membrane if Cl^- is secreted actively. Extrusion of Na^+ from the cell, and therefore maintenance of the favorable Na^+ electrochemical potential gradient, occurs by the ouabain-sensitive ($Na^+ + K^+$)-stimulated ATPase (i.e., primary active Na^+ transport) located within the basolateral membrane. Epithelia that exemplify NaCl cotransport absorption include prawn intestine,[1] flounder intestine,[38,47] sculpin intestine,[94] marine eel intestine,[160] flounder urinary bladder,[145] trout urinary bladder,[119] *Necturus* gallbladder,[44,81] *Necturus* proximal tubule,[165] bullfrog small intestine,[9,143] frog skin,[135,180] bovine rumen,[26] rat colon,[17] rabbit gallbladder,[48] rabbit ileum,[136] and human intestine.[169] Epithelia in which Na^+-coupled Cl^- secretion has been demonstrated include killifish operculum,[29] pinfish gills,[45] shark rectal gland,[156] frog stomach,[150] frog cornea,[23,184] rabbit ileum,[137] and dog trachea.[4] In these systems Na^+ is thought to be actively recycled at the basolateral membrane by the Na^+ pump, whereas Cl^- moves energetically downhill from cytosol to the mucosa via a cAMP-enhanced Cl^- conductance.[116] The third widely accepted epithelial Cl^- transport process is also secondarily active and involves Cl^-/anion antiport and is found, for example, in anal papillae of mosquito larvae,[166] fish gills,[36,110,128] frog skin,[180] urodele intestine,[84] turtle bladder,[126] rat intestine,[95] rabbit colon,[50] and human small intestine.[169] The energy source for this process is unknown, but it has been suggested that uphill Cl^- transport is energized by a favorable downhill electrochemical potential gradient for the counter anion.[49]

However, a considerable amount of Cl^- transport data has accumulated in the transport literature that does not conform to any of the three well-established models described above. For instance, Hanrahan and Phillips[86] have provided evidence for an electrogenic Cl^- accumulative mechanism located in the mucosal membrane of locust rectal epithelium. This mechanism is activated and stimulated directly by K^+ and is also independent of Na^+ and HCO_3^-. Observations of plant cell membranes[80,91] as well as bacterial membranes[186] have yielded Cl^--pump activity and associated Cl^- accumulation that are inconsistent with the three models for Cl^- transport described previously (see above). Perhaps the strongest and most compelling evidence for an ATPase primary active transport mechanism of Cl^- resides with the observations of Gerencser[66,68,77] and Inagaki,[98,155] who have characterized Cl^--ATPase activity and ATP-dependent Cl^- transport in the same plasma membrane system as well as reconstituting these activities in a liposome system.[66,77,100,185] Indeed, the speculation by Frizzell et al.,[49] Schultz,[152] and DePont and Bonting[32] that Cl^--stimulated ATPases are not involved in biological Cl^- transport may have been too presumptuous and premature considering the recent possible evidence to the contrary.

II. EXISTENCE AND GENERAL PROPERTIES OF ANION (Cl^-)-ATPases

A. Definition of a "Pump" or a Primary Active Transport Mechanism

A primary active transport mechanism for a particular solute is a process that moves that solute up its thermodynamic gradient

by its direct linkage with a source of free energy. The source of metabolic free energy may arise from the capture of photons, the movement of electrons, or the hydrolysis of high-energy phosphorylated intermediates such as ATP. If ATP is the source of metabolic free energy, then the "pump," or primary active transport mechanism, is termed an "ion-motive ATPase" or an "ion-transport ATPase." Therefore, an ion-transport ATPase is an enzyme responsible for the primary coupling of ATP hydrolysis to ion movement across a biomembrane. Ion movement is vectorial; likewise, ATP hydrolysis is vectorial at the molecular level; but in the absence of a membrane-transport ATPase assembly, the vectoriality is lost and the hydrolysis is scalar in bulk solution.[109] The role of a transport ATPase, then, is not only to catalyze ATP hydrolysis but to translate the ion movement inherent in the vectorial hydrolysis reaction into a net flux of an ionic species across a membrane. The definition of primary coupling between ATP hydrolysis and ion movement is made explicit by the cross coefficient, R_{kr}, in Equation 1 a general flux equation from nonequilibrium thermodynamics.[109]

$$J_k = \frac{\Delta\bar{\mu}_k}{R_{kk}} - \Sigma\frac{R_{kj}}{R_{kk}}J_j - \frac{R_{kw}}{R_{kk}}J_w - \frac{R_{kr}}{R_{kk}}J_r \quad (1)$$

The term $\Delta\bar{\mu}_k/R_{kk}$ asserts that the flux of an ionic species, J_k, is driven by and is proportional to a difference in the electrochemical potential of that species across a membrane, $\Delta\bar{\mu}_k$, it being understood that this difference has two components, a chemical activity difference given by $RT\Delta \ln a_k$ and an electrical potential difference given by $zF\Delta\Psi$. The straight coefficient, R_{kk}, is a resistance term; that is, a coefficient that converts the proportionality to an equality. A flux coupled solely to $\Delta\bar{\mu}_k$ is universally accepted as being a 'passive' flux. We will consider a flux coupled to the flow of any other solute, J_j, ($R_{kj} \neq 0$) or a flux coupled to the flow of water, J_w, ($R_{kw} \neq 0$) to be secondary to the flow of the other solute or of water. By primary active ion transport we mean the coupling of an ion movement directly to the flow of a chemical reaction, J_r; for such a flux the cross coefficient in the last term, $R_{kr} \neq 0$. Such a primary active ion flux is coupled to a reaction flux, in this case ATP hydrolysis, by mechanisms whose structural orientation within a biomembrane is crucial. In summary, the cross coefficient, R_{kr}, can be viewed as a formal expression of an ion-transport ATPase.

B. P-Type ATPases

A major functional class of membrane-bound enzymes includes those categorized as primary active transporters and called ATPases because they catalyze the transport of ions against an electrochemical potential by reactions linked directly to the hydrolysis of ATP. The ATPases that actively transport cations have been extensively studied and have been categorized by Pedersen and Carafoli[139] into three classes: F-type ATPases (F-ATPases), V-type ATPases (V-ATPases), and P-type ATPases (P-ATPases). The F-ATPases are located in bacterial plasma membranes, inner mitochondrial membranes, and thylakoid membranes of chloroplasts, and they operate as ATP synthases, synthesizing ATP from adenosine diphosphate (ADP) and inorganic phosphate using energy derived from electrochemical gradients of protons.[5] Using reaction mechanisms analogous to F-ATPases, the V-ATPases utilize ATP to create proton electrochemical gradients; they are ubiquitously distributed in eukaryotic vacuoles, lysosomes, archaebacteria, and are also present in plasma membranes of various animal tissues.[88]

The P-ATPases are broadly distributed, active cation translocators having the distinctive feature of forming a covalent acylphosphate-enzyme intermediate (hence, the P-designation) during the cycle of ATP hydrolysis and cation translocation.[139,163] Among this class of ATPases are the Ca^{2+}-ATPases of the plasma membrane, sarcoplasmic reticulum and endoplasmic reticulum, the H^+-ATPase of yeast and plants, the K^+-ATPase of bacteria, the Na^+/K^+-ATPase of animal cell plasma membranes, and the H^+/K^+-ATPase of gastric parietal cells. Because of the formation of phosphoenzyme intermediates, the enzymatic cycle of P--ATPases can be divided into steps that include a kinase activity, by which an aspartate residue on the enzyme is phosphorylated, and a phosphatase activity, by which the phosphoenzyme is dephosphorylated.[163] Another common feature of these ATPases is their inhibition by submicromolar concentrations of vanadate, acting as a tightly binding phosphate analog.[24,138] Furthermore, during the enzymatic cycle, P-ATPases characteristically exhibit two phenomenologically and structurally distinct conformations, E_1 and E_2, that have distinct kinetic variables.[105] For this reason, the P-ATPases are also called E_1-E_2 ATPases.

In addition to their functional similarities, P-ATPases have a number of structural homologies, belonging to a common large gene family. All members have a principal peptide of approximately 100 kDa, designated as the catalytic (sub)unit because it contains the site for ATP binding and phosphorylation.[139] The high degree of sequence homology for amino acids within the ATP binding site and the phosphorylation site attests to the highly conserved nature of protein domains that interact with ATP. Some disagreement remains as to the exact location, and even the number, of transmembrane segments;[100] the specific locations for the binding of cations and the paths for their transport are far from resolved.

C. Characteristics of Anion ATPases

Since the time Durbin and Kasbekar[40] first demonstrated anion-stimulated ATPase activity in a microsomal fraction of frog gastric mucosa, there has been little question as to the existence of, at least, the biochemical manifestation of the enzyme. The distribution of anion-stimulated ATPase activity seems to be as widely distributed throughout biology as the number of different plants and animals studied.[32,63,153]

Anion-stimulated ATPase activity, and therefore possibly Cl^- pump existence, has been demonstrated in both microsomal and mitochondrial fractions of many tissues (Table 1) in which HCO_3^-, Cl^-, or H^+ transport occurs, suggesting a transport function for this enzyme. DeRenzis and Bornancin[35] demonstrated the existence of a Cl^-/HCO_3^--stimulated ATPase in goldfish gill epithelia. It was not until this documentation in 1977 that HCO_3^--stimulated ATPase activity was linked with possible primary active Cl^- transport, because Cl^- stimulation of this enzyme had not been demonstrated previously.

As the name of the enzyme implies, it is stimulated directly by anions, especially HCO_3^- and Cl^-. Bicarbonate stimulation of the enzyme has occupied the predominant focus of attention, primarily because of cellular acid-base implications and also because of possible simultaneous proton secretion in gastric mucosal systems.[32,33] However, HCO_3^- can be replaced by several other anions, especially Cl^- and the oxyanions such as arsenate, arsenite, borate, selenite, sulfate, and sulfite.[18,157,168,178] As can be surmised, however, there are consid-

TABLE 1
Some Biological Tissues in Which Anion$^-$-Stimulated ATPase Activity Has Been Localized to Cellular Plasma Membranes or Microsomal Fractions

Tissue	Species	Ref.
	Plants	
Cell membrane	Algae	97
Salt gland cell membrane	Salt marsh	90
	Animals	
Brain	Rat	155
Embryo	Sea urchin	132
Gastric mucosa	Dog	150
	Frog	40
	Lizard	33
	Necturus	183
	Rabbit	173
	Rat	164
Gills	Goldfish	35
	Eel	19
	Trout	20
		16
	Fiddler crab	31
	Blue crab	125
Intestine	Eel	133
	Rat	96
	Aplysia	74
Kidney	Dog	150
	Frog	72
	Mouse	72
	Rabbit	127
	Rat	114
Lens	Cow	106
Liver	Rat	103
Mantle	Oyster	181
Midgut	Moth	168
Pancreas	Cat	159
	Dog	157
	Rat	174
Placenta	Human	21
Rectum	Larval dragonfly	117
	Locust	124
Salivary gland	Dog	102
	Rabbit	157
	Rat	178
Seminiferous tubules	Rat	154
Spinal motoneurons	Rat	98
Uterus	Rat	101

erable differences in effectiveness of the various anions in different tissues.[172] As an extreme example, glucaronate stimulates ATPase activity in lizard gastric mucosa,[33] whereas it inhibits, presumably, the same enzyme in frog gastric mucosa.[107] As emphasized by Schuurmans Stekhoven and Bonting,[153] this species and tissue variability may very well be caused by affinity differences of the various anions for the enzyme.

ATP is the preferred substrate for the anion-stimulated ATPase, with an optimal Mg^{2+}/ATP ratio ranging from 0.5 to 2.0.[159,172,173,174] Guanosine triphosphate (GTP) and inosine 5′-triphosphate (ITP) are less preferred substrates than ATP for the anion-stimulated ATPase, whereas uridine triphosphate (UTP) and cytidine triphosphate (CTP) are slightly hydrolyzed or not hydrolyzed at all by the enzyme.[18,159]

The divalent cation Mg^{2+} is absolutely required for anion-stimulated ATPase activity, but inhibits at high concentrations,[40,74,77] as are also the cases for the cation-stimulated enzymes $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase. Mn^{2+} can substitute for Mg^{2+} in the gastric mucosal enzyme,[149] but does so to a lesser extent in the pancreatic enzyme.[158,159,174]

Generally, Na^+ or K^+ has little or no effect on the anion-ATPase activity,[40,159] but K^+ was shown to have a stimulatory effect on the enzyme in rat salivary glands,[178] whereas NH_4^+ appears to inhibit anion-stimulated ATPase activity[149] in frog gastric mucosa.

III. LOCATION

One of the most controversial issues regarding Cl^--stimulated ATPase activity is its site or anatomical localization within the microarchitecture of cells. It appears that Cl^--stimulated ATPase activity resides in both mitochondrial and microsomal fractions[32] of cell homogenates. However, DePont and Bonting[32] and Schuurmans Stekhoven and Bonting[153] have categorically stated that microsomal or plasma membrane localization of this enzyme is entirely due to mitochondrial contamination. Hence, the dispute. If Cl^--stimulated ATPase activity is exclusively of mitochondrial origin it is extremely difficult to conceive how it could drive net Cl^- movement across plasma membranes. Therefore, the Cl^--stimulated ATPase should play no direct role in transcellular Cl^- transport, but could function, in some capacity, in intracellular Cl^- transport. On the other hand, if the Cl^--stimulated ATPase is located in the plasma membrane, then primary Cl^- transport by this enzyme would be analogous to the $(Na^+ + K^+)$-stimulated ATPase that mediates net transport of Na^+ and K^+ across plasma membranes.[161]

Mature rabbit red cells do not contain mitochondria; hence, any Cl^--stimulated ATPase activity found in these cells[39,104,173] must be localized within the plasma membrane. However, this enzyme, which is primarily stimulated by HCO_3^-, is very different from the enzyme that is found in other tissues.[175] For example, Na^+ stimulates the enzyme, as do thiocyanate and acetazolamide.[104] Cations are not known to stimulate Cl^--stimulated ATPase in other tissues, and thiocyanate is a potent inhibitor of the enzyme in almost all tissues studied.[153] Rather than stimulating, sulphite inhibited the enzyme activity, and HCO_3^- had a relatively small stimulatory effect. These properties are widely divergent from those observed for anion-stimulated ATPase found in other tissues.[32] Because Ca^{2+} stimulated the ATPase activity and known inhibitors of $(Ca^{2+} + Mg^{2+})$-ATPase activity (such as EGTA, chlorpromazine, and ruthenium red) inhibited the anion-stimulated ATPase activity, it was concluded that the anion-stimu-

lated enzyme activity is part of the (Ca^{2+} + Mg^{2+})-stimulated ATPase of the red cell membrane, rather than representing a separate, functional anion-stimulated ATPase.[175] Au[11] confirmed these findings by showing that calmodulin stimulated, in parallel, both red cell (Ca^{2+} + Mg^{2+})-ATPase and anion-stimulated ATPase activity and that the activities of both enzymes were depressed by an inhibitory protein present in pig red cells. These results suggested that the observed anion-stimulated ATPase activity in red cells is either part of another enzyme system that also requires divalent cations or that this enzyme is structurally and functionally different from those Cl^--stimulated ATPases described previously.[32]

Another perplexing example of Cl^--stimulated ATPase activity possibly coexisting with another enzyme system is that demonstrated in rat enterocyte plasma membranes[96] and the plasma membranes from human placental epithelial cells.[21] The Cl^--stimulated ATPase activity in brush border membranes of these two tissues was relatively low compared with the enzyme activity in other tissues.[32] Specifically, the Cl^--stimulated ATPase in intestinal brush-border membranes could be inhibited by L-phenylalanine and L-cysteine (specific inhibitors of alkaline phosphatase), which suggested that this anion-stimulated enzymatic activity and alkaline phosphatase activity originate from a single, functional enzyme.[96] Supporting this hypothesis was the additional observation that the Cl^--stimulated ATPase showed a pH optimum of 8.5, commensurate with pH optima of alkaline phosphatase preparations,[96] and widely different from the pH optima of 7.5 to 7.6 of Cl^--stimulated ATPase activity found in other tissues.[153] Similar results and a like conclusion were reached for the Cl^--stimulated ATPase found in human placental brush-border membranes.[21] It appears, most likely, that the Cl^--stimulated ATPase activity from these two brush-border membrane preparations is a coexistent property of the alkaline phosphatase enzyme system.

Without question, the primary location of anion- (specifically Cl^-)-stimulated ATPase activity within animal cells appears to be in the mitochondria, that is, a property of the mitochondrial H^+-ATPase.[144] Even though Grisolia and Mendelson[83] presented evidence that anion-stimulated ATPase activity is located within the outer membrane of mitochondria, the major portion is probably located within the inner membrane and is possibly identical to the ATPase involved in oxidative phosphorylation.[120,144] Obviously, the key question that remains is: what is the origin of the Cl^--stimulated ATPase activity of non-mitochondrial organelles? Is it as Van Amelsvoort et al.[172–174] have so strongly stated that all nonmitochondrial organelles that exhibit Cl^--stimulated ATPase activity have been contaminated with the mitochondrial-based enzyme, or is there a true, separate Cl^--stimulated ATPase that is localized within the cellular plasma membranes and, therefore, can possibly act as the prime effector of net Cl^- movement between the intracellular and extracellular milieu?

Van Amelsvoort et al.[172] provided extensive evidence using differential and density gradient centrifugation techniques on epithelia from trout gill, rabbit kidney, and rabbit stomach that most, if not all, anion-stimulated ATPase activity is of mitochondrial origin. Their speculative conclusions negated any plasma membrane anion-stimulated ATPase localization found in other studies[111,157,158,178] on the basis that the results from these studies were possibly artefactual due to improper homogenization and density gradient centrifugation techniques. They stated that excessive or 'drastic' homogenization may inactivate the mitochondrial anion-stimulated ATPase by release of the endogenous mitochondrial

inhibitory protein;[25] therefore, this effect would amplify, in a relative sense, mitochondrial contamination observed in nonmitochondrial organelles. However, they did not comment why the mitochondrial inhibitory protein would not also inactivate the mitochondrial contaminant, anion-stimulated ATPase found in nonmitochondrial organelles. Surprisingly, in the same study, Van Amelsvoort et al.[172] observed low cytochrome oxidase activity in presumed mitochondrial-rich fractions of rabbit kidney and stated that cytochrome oxidase was either specifically inactivated, or that loss of the mitochondrial inhibitory protein led to an exaggerated anion-stimulated ATPase activity in these fractions. They did not present data nor did they speculate on how these mechanisms were actuated in light of the apparent contradiction based on the argument that they put forth for 'drastic' homogenization effects. They also stated that 'drastic' homogenization techniques may yield extremely small submitochondrial particles, which may not reach their equilibrium position in normal empirically determined times of density gradient centrifugation, which could also account for erroneous plasma membrane localization of anion-stimulated ATPase activity. It had been the preceding studies that negated any interpretation, other than anion-ATPase being a property of mitochondrial H^+-ATPase, that stultified progress in this most complex research area for a period extending from the early 1970s through the mid-1980s.

Several other reports have supported the contention that Cl^- (anion)-stimulated ATPase activity resides exclusively in the mitochondria.[83,92,101,103,112,173] This is an absolute possibility in tissues whose sole function is utilizing the anion-stimulated ATPase in the production of energy for cellular maintenance. However, through adaptational demands, other specialized groups of cells (tissues) may possibly need the Cl^--stimulated ATPase for other cellular functions, such as transducing metabolic energy into net osmotic (Cl^-) movement between the intracellular and extracellular milieu in order to maintain cellular homeostasis. This supposition necessitates the plasma membrane localization of the Cl^- transport process. As suggested earlier (see above), there are numerous examples of those tissues that transport Cl^- whose processes of transfer have been modeled mechanistically, but thermodynamically have not been rigorously defined or tested. Invoking a cellularly active Cl^- transport mechanism on energetic grounds justifies the search for such a process in the one cellular organelle that regulates the transfer of material and information (Cl^-) between the external world and intracellular contents, the plasma membrane.

The plasma membrane that surrounds the cell periphery of renal proximal tubule epithelial cells consists of both basolateral and luminal aspects, the luminal membrane being constituted by microvilli (brush border). These asymmetrical membranes can be separated by differential centrifugation and free-flow electrophoresis techniques. Kinne-Saffran and Kinne,[113] using free-flow electrophoresis of rat kidney cortex, demonstrated that HCO_3^--stimulated ATPase comigrated with alkaline phosphatase activity, but was separated from ($Na^+ + K^+$)-stimulated ATPase activity, which is a marker enzyme for the basolateral membranes. These results suggested that the luminal membrane of rat proximal tubule epithelial cells contains a HCO_3^--stimulated ATPase. Similar conclusions were reached by Liang and Sacktor[127] for brush-border membrane preparations from rabbit kidney cortex.

However, because of valid, stringent, criticism of these experiments by Van Amelsvoort et al.,[172,173] who championed the contention that mitochondrial contamination of brush border anion-stimulated

ATPase could not be ruled out, Kinne-Saffran and Kinne[114] reinvestigated the problem of a nonmitochondrial anion-stimulated ATPase in rat kidney. This investigation proved to be one of the early defining studies in determining the plasma membrane existence of nonmitochondrial anion-stimulated ATPase activity.

Kinne-Saffran and Kinne[114] proceeded to isolate simultaneously under identical conditions both a plasma membrane fraction, rich in brush-border membranes, and a mitochondrial fraction. This was done to avoid different types of chemical and/or physical perturbations of the enzyme activities in the two fractions. Because it was observed that both mitochondrial and brush-border membrane fractions contained a Mg^{2+}-ATPase that could be stimulated by both Cl^- and HCO_3^-, the critical question of whether the ATPase activity observed in the brush border membrane fraction could be accounted for by part of the total ATPase activity in the mitochondria was answered by the following results: the specific activity of the brush border membrane (Mg^{2+} + anion)-ATPase was two to three times that of the mitochondrial enzyme, and there was a direct relationship between the enrichment of the ATPase and the reduction of succinic dehydrogenase in the brush-border membrane fraction. This disparity became greater when the mitochondrial inhibitory protein[25] was added to each fraction and, it was strikingly clear that only the mitochondrial enzyme was inhibited, whereas no effect was observed on the brush-border membrane enzyme. Calculations by Kinne-Saffran and Kinne[114] revealed that 2% mitochondrial contaminant anion-stimulated ATPase contributed to the total anion-stimulated ATPase activity observed in the brush-border membrane fraction. L-*p*-Bromotetramisole, an inhibitor of alkaline phosphatase activity, was added to both brush-border membrane and mitochondrial fractions.[114] All alkaline phosphatase activity in the brush-border fraction was inhibited; however, virtually no effect was observed on the anion-stimulated ATPase activities of either the plasma membrane or mitochondrial fractions. This observation made it quite unlikely that the measured activity of anion-stimulated ATPase in the brush border was due to a hydrolytic action of alkaline phosphatase on ATP.

Kinne-Saffran and Kinne[114] looked for other means to distinguish between the brush-border membrane and mitochondrial anion-stimulated ATPases. For example, they utilized the difference in chemical compositions of the mitochondrial and plasma membranes. In the inner mitochondrial membrane, there exists an adenine nucleotide translocator that is present solely in mitochondria, and that is responsible for the transfer of ATP across the membrane. This system can be blocked by atractyloside, thereby preventing ATP transport to the active site of the mitochondrial anion-stimulated ATPase.[115] Kinne-Saffran and Kinne[114] demonstrated that atractyloside exclusively inhibited the mitochondrial anion-stimulated ATPase activity and had no effect on the brush border anion-stimulated ATPase activity (Table 2). This observation strongly suggested that the anion-stimulated ATPase in the brush border was not of mitochondrial origin.

Besides the noted difference in functional proteins between plasma membranes and mitochondrial membranes, there is a great difference in the amount and types of lipids present in the two types of membranes.[85,172,173] Brush-border membranes are rich in cholesterol, whereas mitochondrial membranes are relatively poor in cholesterol.[85] Based on this difference in cholesterol composition, Kinne-Saffran and Kinne[114] used filipin, a polyene antibiotic, for further functional differentiation between the mitochondrial and brush-border anion-

TABLE 2
The Effect of Carboxy-Atractyloside on the Mg^{2+}-ATPase and the (Mg^{2+} + HCO_3^-)-ATPase Activity in the Mitochondrial Fraction and in the Brush Border Membrane Fraction

	Added ions				Mg^{2+} = HCO_3^-		
	Mg^{2+}	% of control	Mg^{2+} + HCO_3^-	% of control	ΔHCO_3^-	% of control	Mg^{2+}
Mitochondrial fraction							
Control	15.9 ± 0.8	100	30.1 ± 1.2	100	14.2 ± 0.9	100	1.89
50 μ*M* carboxy-atractyloside	7.6 ± 0.5	47.8	14.6 ± 0.6	48.5	7.0 ± 0.6	49.3	1.92
100 μ*M* carboxy-atractyloside	7.6 ± 0.5	47.8	14.9 ± 0.3	49.5	7.3 ± 0.3	51.4	1.96
Brush border membrane fraction							
control	57.3 ± 1.9	100	71.5 ± 2.1	100	14.2 ± 0.8	100	1.25
50 μ*M* carboxy-atractyloside	57.1 ± 2.0	99.7	71.7 ± 2.1	100.3	14.6 ± 0.8	102.8	1.26
100 μ*M* carboxy-atractyloside	68.2 ± 1.6	101.6	72.7 ± 1.9	100.7	14.5 ± 0.7	102.1	1.25

Note: The experiments were performed with intact mitochondria or freshly prepared brush border membranes, respectively. The mean values of five experiments are shown. The enzyme activities are given in μmol h^{-1} mg^{-1} protein. All differences were statistically significant at the level of $P < 0.001$. Reprinted with permission from Kinne-Saffran and Kinne.[114]

stimulated ATPases. Filipin interacts with cholesterol in the membrane and causes perturbations of the lipid surrounding the enzyme.[30] Filipin was shown to inhibit the anion-stimulated ATPase activity of the brush-border membrane fraction, whereas it had no effect on the mitochondrial enzyme (Table 3). Taken *in toto*, Kinne-Saffran and Kinne[114] provided the first strong evidence for the existence of a separate, plasma membrane-bound, anion-stimulated ATPase that could be distinguished from the same enzyme of mitochondrial origin.

DeRenzis and Bornancin[35] reported anion ($Cl^- + HCO_3^-$)-stimulated ATPase activity in the epithelium of goldfish gills. The enzymatic activity was predominantly localized within the plasma membranes, in the absence of mitochondrial contamination. Bornancin et al.[20] demonstrated the same activity in the plasma membranes of trout gill epithelium with virtually no mitochondrial contaminant anion-stimulated ATPase activity being present. These results sharply disagreed with those of Van Amelsvoort et al.,[172–174] who concluded that all anion-stimulated ATPase activity observed in trout gill plasma membranes originated from mitochondrial contamination. These authors advanced the argument that the increase in the ratio of anion-stimulated ATPase activity and succinic dehydrogenase activity of the plasma membranes and mitochondria, respectively, was due to the loss of mitochondrial inhibitory protein. This speculative conclusion is not well founded when based upon the type of separation described by Horstman and Racker[93] that is needed to extract the mitochondrial inhibitory protein.

Cole[28] described HCO_3^--stimulated ATPase activity in plasma membranes of rat kidney cortex epithelial cells. Enrichment of the plasma membrane anion-stimulated ATPase activity also resulted in a 10-fold diminution of succinic dehydrogenase activity, suggesting the existence of an integrated anion-stimulated ATPase in the plasma membranes of rat renal cortical cells that is separate from the mitochondrial-based enzyme. Similar results were shown for plasma membranes from renal medullary epithelial cells.[150] In fact, after these authors had demonstrated that mitochondrial inhibitory protein had virtually no effect on the anion-stimulated ATPase located in the apical plasma membrane, they used the ATPase as a marker enzyme for the apical membrane.

Komnick, Schmitz, and Hinssen[117] described Cl^--stimulated ATPase activity in both mitochondrial and plasma membrane fractions of larval dragonfly rectal epithelial cells. They demonstrated an increase in Cl^--stimulated ATPase activity in the plasma membrane fraction during the preparative procedure. This was accompanied by a decrease in mitochondrial contamination. This study supports the hypothesis of differentially localized Cl^--stimulated ATPases.

Anion (Cl^--HCO_3^-)-stimulated ATPase activity was also observed in plasma membrane fractions of fiddler crab (*Uca minax*) gill epithelium.[31] Although the authors were unable to completely eliminate mitochondrial anion-stimulated ATPase contamination from the plasma membrane fractions, calculations of the maximal activity of anion-stimulated ATPase attributed to mitochondrial fragments amounted to 33% of that observed. Similar results were obtained by Wheeler and Harrison[181] for anion-stimulated ATPase localization in clam mantle epithelium. Lee[125] demonstrated an anion-stimulated ATPase in purified plasma membranes of blue crab (*Callinectes sapidus*) gill epithelium that could be differentiated from its mitochondrial counterpart.

Perhaps the strongest evidence for the existence of Cl^--ATPase activity in a plasma membrane system free from any possible mitochondrial contaminant ATPase was that

TABLE 3
The Effect of Filipin on the Mg^{2+}-ATPase and the (Mg^{2+}+HCO_3^-)-ATPase Activity in the Mitochondrial Fraction and in the Brush Border Membrane Fraction

	Added ions				$Mg^{2+} = HCO_3^-$		
	Mg^{2+}	% of control	$Mg^{2+} + HCO_3^-$	% of control	ΔHCO_3^-	% of control	Mg^{2+}
Mitochondrial fraction							
control	21.3 ± 1.7	100	39.8 ± 2.5	100	18.5 ± 1.2	100	1.87
35 μ*M* filipin	21.8 ± 2.5	102.3	39.9 ± 2.3	100.3	18.1 ± 1.2	97.8	1.83
70 μ*M* filipin	20.8 ± 1.4	97.6	40.0 ± 1.4	100.5	19.2 ± 1.8	103.8	1.92
Brush border membrane fraction							
Control	60.1 ± 2.0	100	75.0 ± 1.8	100	14.9 ± 1.3	100	1.25
35 μ*M* filipin	35.8 ± 0.9	59.6	48.0 ± 1.6	64.0	12.2 ± 1.0	81.9	1.34
70 μ*M* filipin	23.3 ± 0.5	38.8	32.9 ± 1.4	43.9	9.6 ± 1.0	64.6	1.41

Note: The experiments were performed with freeze-thawed mitochondria or freeze-thawed brush border membranes, respectively. The mean values of six experiments are shown. The enzyme activities are given in μmol h^{-1} mg^{-1} protein. The differences were statistically significant at the level of $P < 0.001$, except for the case marked with an asterisk, where $0.005 < P < 0.001$. Reprinted with permission from Kinne-Saffran and Kinne.[114]

by Gerencser and Lee.[72–74] They presented evidence that indicated that purified basolateral membranes (BLM) of *Aplysia* foregut absorptive cells contain Cl^--ATPase activity. Their finding that the BLM subcellular membrane fraction had a high specific activity in $(Na^+ + K^+)$-ATPase, but had no perceptible cytochrome c oxidase activity and a significantly reduced succinic dehydrogenase activity, supported this conclusion. The observation that there was very little nicotinamide-adenine dinucleotide phosphate (NADPH)-cytochrome c reductase activity in the membrane fraction suggested that the BLM in this fraction were also relatively free from endoplasmic reticulum and Golgi body membrane contamination.[131] The failure of oligomycin to inhibit Cl^--ATPase activity in the BLM fraction was also consistent with the nonmitochondrial origin of the Cl^--ATPase. Supporting this contention was the corollary finding that oligomycin inhibited mitochondrial Cl^--stimulated ATPase activity. The finding that efrapeptin, a direct inhibitor of mitochondrial F_1-ATPase activity,[22] significantly inhibited Mg^{2+}-ATPase activity in the mitochondrial and not in the BLM fraction[74] unequivocally supported the notion that the plasma membrane fraction was pure and was free from mitochondrial contamination. Additionally, Gerencser and Lee[74] showed that vanadate (an inhibitor of only P-type ATPases) inhibited Cl^--ATPase activity in the purified BLM fraction and not in the mitochondrial fraction. Taken together, all of these observations strongly supported the hypothesis that Cl^--stimulated ATPase activity exists in, at least, one subcellular locus other than mitochondria. It appears that in numerous biological cells, which transport Cl^-, Cl^--stimulated ATPase activity forms an integral part of the plasma membrane.[60,61,63,72,78]

IV. FUNCTION OF ANION-ATPases

To assign a direct role of Cl^- or HCO_3^- transcellular transport to an ATPase, the enzyme should be shown to be an integral component of the plasma membrane. The energy for active transport of Cl^- or HCO_3^- can, in principle, thus be obtained from the hydrolysis of ATP. Therefore, the following question can be asked: Is the anion-stimulated ATPase identical with a 'pump' for anions? The following discussion deals with this controversial question.[32,56,57,152]

Counter-transport of Cl^- and HCO_3^- has been reported in the gills of goldfish[36,130] and trout.[110] This exchange is inhibited by thiocyanate.[34,43,111] The Cl^-/HCO_3^- exchange process has also been reported in molluscan neurones[148,167] and mouse soleus muscle,[2] which is sensitive to 4-acetamido-4′-isothiocyanostilbene-2,2′ disulphonic acid (SITS) and is not inhibited by thiocyanate in mouse soleus. It has also been reported in numerous epithelia[55,57] that this anion exchange process exists and is sensitive to the stilbene derivatives. The stilbene-sensitive counter-transport or antiport mechanism does not seem to require ATP and, therefore, in all probability is not an ATPase.[147]

It was not until the following observations that HCO_3^--stimulated ATPase activity was linked with Cl^- pumping because no Cl^- activation of this enzyme had been observed. DeRenzis and Bornancin[35] were the first to demonstrate the membrane presence of a (Cl^-/HCO_3^-)-stimulated ATPase in goldfish gill epithelium and suggested that the enzyme could participate in the branchial Cl^-/HCO_3^- exchange mechanism. Bornancin et al.[19] confirmed these results in freshwater eel gill epithelium, as did Bornancin et al.[20] in freshwater trout gill epithelium. Kinetic studies in these three gill epithelial systems strongly suggested that a (Cl^-/HCO_3^-)-stimu-

lated ATPase is involved in the Cl^-/HCO_3^- exchange mechanism and therefore in the acid-base regulation of freshwater fish. These authors reported a parallelism between the affinities of the ATPase for Cl^- and both the Cl^- affinity for the gill transport mechanism and the Cl^- influx rate. The affinity constants for the Cl^--stimulated ATPase were 1.0, 5.9, and 23.0 meq/L for the goldfish,[35] freshwater trout,[20] and freshwater eel[19] gill epithelium, respectively. The affinity of Cl^- for the transport systems *in vivo* was 0.07, 0.25, and 1.3 meq/L for the goldfish,[35] freshwater trout,[20] and freshwater eel[19] gill epithelium, respectively, whereas the corresponding maximal Cl^- influxes were 55.0, 19.6, and 0.36 μeq/h/100 g. In addition, the finding that the Cl^- activation of anion-stimulated ATPase activity was inhibited by thiocyanate[35] was consistent with transport studies that showed that Cl^- influxes were inhibited by thiocyanate.[34] These studies on gill epithelium strongly supported the hypothesis that the Cl^--stimulated ATPase is involved in gill anion exchanges that are related to mineral and acid-base homeostasis in freshwater fish.

The fiddler crab gill has been shown to actively absorb Cl^- from low salinities[13] and actively extrude Cl^- in high-salinity media.[12] In concert with these findings, DePew and Towle[31] demonstrated the existence of an anion-stimulated ATPase in the gill cell plasma membrane of fiddler crab and suggested that this enzyme is so situated with its environment that it is highly accessible to Cl^- and HCO_3^-, and thus may play a direct role in active Cl^-/HCO_3^- exchange.

Lee[125] creatively approached the etiological question concerning the correspondence between transport and anion-stimulated ATPase activity by the following logic. After it was established that anion-stimulated ATPase activity existed in the plasma membrane of blue crab gill epithelium, the animals were adapted to low salinities. This thinking presumed that Cl^-/HCO_3^- exchange should increase under these osmotic stressful conditions; therefore, this activity should be reflected in an increase in the activity of anion-stimulated ATPase activity. This was indeed the case and Lee[125] suggested that anion-stimulated ATPase activity appears likely to play an important role in anion transport for osmoregulatory and/or acid-base homeostasis in marine organisms.

Komnick et al.[117] reported the presence of (Cl^-/HCO_3^-)-stimulated ATPase activity in plasma membranes of larval dragonfly rectum. The Cl^--stimulated ATPase activity was inhibited by thiocyanate as was the Cl^- influx into the rectal epithelia. These results suggested the possible existence of an ATPase-mediated, active Cl^- transport mechanism located in the plasma membrane of larval dragonfly rectal epithelial cells that could participate in osmoregulatory responses for these cells.

In the eel (*Anguilla japonica*) intestine, electrophysiological experiments have shown that active transport of Cl^- coupled with water transport markedly increases during seawater adaptation.[6,7] The observed increase in Cl^- absorption raised the question of an associated increase in activity of an enzyme contributing to the transport process. It was demonstrated by Morisawa and Utida[133] that anion-stimulated ATPase activity existed in an oligomycin-insensitive, thiocyanate-sensitive membrane fraction of eel intestinal enterocytes that was also relatively deficient of cytochrome oxidase activity. Seawater adaptation increased the enzyme activity commensurate with changes in Cl^- and water transport. From these considerations, these authors concluded that the anion-stimulated ATPase played a direct role in Cl^- transport and, secondarily, in water absorption in the eel intestine.

The hindgut of the desert locust possesses an unusual chloride transport sys-

tem.[86,87] The isolated locust rectum absorbs chloride from the mucosal (lumen) to the serosal (haemolymph) side in the absence of an electrochemical potential gradient. Net chloride transport persists in nominally Na-free or $HCO_3(CO_2)$-free saline, is insensitive to normal inhibitors of NaCl cotransport and anion exchange, and is independent of the net electrochemical gradient for sodium across the apical membrane. However, active chloride transport is strongly dependent on mucosal potassium. Chloride entry across the apical membrane is active, whereas the net electrochemical gradient across the basal membrane favors passive Cl^- exit from the cell. Although mucosal potassium directly stimulates "uphill" chloride entry, there is no evidence for coupled KCl cotransport, nor would coentry with potassium be advantageous energetically. To determine if active Cl^- transport across rectal epithelia might be due to an anion-stimulated ATPase, a microsomal fraction was obtained by differential centrifugation.[124] Microsomal ATPase activity was stimulated in the following sequence: sulphite > bicarbonate > chloride. Maximal ATPase activity was obtained at 25 m*M* HCO_3^- or 25 m*M* Cl^-. Thiocyanate inhibited 90% of the anion-stimulated ATPase activity. The microsomal fraction was enriched in the plasma membrane markers, leucine aminopeptidase, alkaline phosphatase, 5′nucleotidase, and gamma-glutamyltranspeptidase, and had little contamination from the mitochondrial enzymes, succinate cytochrome c reductase, and cytochrome oxidase. Microscopic examination confirmed that the BLMs were associated with mitochondria following differential centrifugation, whereas the microsomal fraction contained little mitochondrial contamination. These results indicate the presence of an anion-stimulated ATPase activity that could be responsible for active Cl^- transport across locust rectum.

In cultured cells derived from isolated micromeres of sea urchin eggs, Cl^-/HCO_3^--ATPase activity was found in the plasma membrane and the microsomal fractions before and after the initiation of spicule formation.[132] After initiation, the skeletal vacuole fraction was obtained from subcellular structures containing spicules. Cl^-/HCO_3^--ATPase in the skeletal vacuole membrane possibly mediates HCO_3^- transport into the vacuoles to supply HCO_3^- for spicule formation.

An anion-stimulated ouabain-insensitive Mg^{2+}-ATPase activity has been found in fresh homogenates prepared from capsules and epithelia of bovine lenses.[106] Approximately equal activity was observed in the presence of HCO_3^- or of Cl^-. The stimulation of each anion obeyed saturation kinetics, with an optimum at approximately 20 m*M* Cl^- or HCO_3^-. In keeping with several other tissues, the diuretic drug ethacrynic acid was inhibitory. ATP was the primary substrate for the enzyme, which also shows some activity for GTP and ITP. Little Na^+/K^+-dependent ATPase activity was observed in the fresh homogenate, but it increased in lyophilized preparations. In contrast, the lyophilized preparations showed no anion-dependent ATPase activity. It was postulated that active HCO_3^- transport in the lens may be mediated by this anion-dependent ATPase.

Halorhodopsin,[89,121] one of the retinal proteins in the cytoplasmic membrane of halobacteria, is an inward-directed light-driven electrogenic pump for chloride ions that generates an inside-negative membrane potential similar to that of bacteriorhodopsin, which transports protons out of the cell interior. However, the physiological role of halorhodopsin might be not only to generate a transient proton-motive force on illumination, but also to maintain cell volume.[130] This is because in these organisms the high (several molar) external NaCl concentra-

tion in the medium is balanced mostly by intracellular KCl, and although the replacement of Na^+ with K^+ can be accomplished, as in many other systems, by a combination of a H^+/Na^+ antiporter[42,122] and electrogenic K^+ uptake,[52,177] the net uptake of Cl^- requires an active accumulation system. Indeed, as with protons in the case of bacteriorhodopsin, a second transport pathway for active Cl^- transport exists in the dark,[41] apparently driven independently, by a proton-motive force. The elements of the foregoing hypothesis can be observed in vesicles prepared from *Halobacterium halobium* cell envelopes containing halorhodopsin.[151] Thus, in the absence of K^+ (e.g., in 3 *N* NaCl), illumination causes the inward flow of Cl^-, which is detectable by direct determination of the accumulated Cl^- in vesicles equilibrated first with Na_2SO_4 or phosphate. When the illumination is started, there is an initial passive influx of protons, which slows as a concentration gradient for protons (inside acid) develops. During this time, Na^+ takes over as the main counterion to the Cl^- movement. Once the proton-motive force approaches zero, the net proton flux ceases, and light will drive the continued uptake of NaCl instead. Indeed, illumination is seen to cause swelling of the vesicles, particularly when gramicidin is added to increase the electrical potential-driven secondary Na^+ uptake.

Halorhodopsin, as with bacteriorhodopsin, requires no other component than the opsin, a small (mol wt about 26,000) integral membrane protein, and the retinal, for the light-driven transport.[121] In both proteins, the retinal is attached to a lysine via a protonated Schiff base. The intimate association of the retinal with various amino acid residues in halorhodopsin is indicated by the fact that the wavelength maximum of the pigment is shifted from 440 nm, that of a protonated retinal Schiff base in solution, to 578 nm. Thus, halorhodopsin, as bacteriorhodopsin, is a purple protein. Absorption of a photon causes the isomerization of the retinal from all-*trans* to 13-*cis*; this initiates a sequence of thermally driven reactions that lead back to the parent pigment in a few tens of milliseconds (the "photocycle"). This characterization provides the first strong evidence for the evidence of a Cl^- pump mechanism residing in bacteria.[121,122] However, as described, this Cl^- pump is powered by photon absorption and not by the hydrolysis of ATP.

The following studies on rat brain motoneurons provided the strongest evidence in vertebrates for the existence and function of a Cl^- pump. Shiroya et al.[155] demonstrated that ethylenediaminetetraacetic acid (EDTA)-treated microsomes prepared from rat brain consisted mainly of sealed membrane vesicles 200 to 500 nm in diameter and were rich in both Cl^--ATPase and Na^+/K^+-ATPase activities. Such Cl^--ATPase-rich membrane vesicles accumulated Cl^- in an ATP-dependent and osmotically reactive manner in the presence of ouabain. The Cl^- uptake was maximally stimulated by ATP with a K_m value of 1.5 m*M*; GTP, ITP, and UTP partially stimulated Cl^- uptake, but CTP, beta, gamma-methylene ATP, ADP, and AMP did not. The ATP-dependent Cl^- uptake was accelerated by an increase in the medium Cl^- concentration with a K_m value of 7.4 m*M*. Such stimulation of Cl^- uptake by ATP was dependent on the pH of the medium, with an optimal pH of 7.4, and also on the temperature of the medium, with an optimal range of 37 to 42°C. Ethacrynic acid dose-dependently inhibited the ATP-dependent Cl^- uptake with a concentration for half-maximal inhibition at 57 μ*M*. *N*-ethylmaleimide completely inhibited and vanadate partially inhibited the ATP-dependent Cl^- uptake. The membrane vesicles did not accumulate H^+ in the Cl^- uptake assay medium. The ATP-dependent Cl^- uptake profile agreed with that of Cl^--ATPase ac-

tivity reported previously.[98,99] More recently, the Cl^--ATPase from rat brain has been reconstituted into liposomes and has been shown to support an ATP-dependent Cl^- uptake.[185] These data collectively strongly supported the idea that Cl^--ATPase in the brain actively transports Cl^- and does so for the partial maintenance of the nerve cell membrane potential.

Gradmann and his colleagues[80] have provided electrophysiological data and ATP synthesis by the Cl^- pump through reversal of Cl^- electrochemical gradients in *Acetabularia* that provided strong evidence for the existence of a Cl^- pump in algae. Buttressing these conclusions were those of Ikeda and Oesterhelt,[97] who showed a Mg^{2+}-ATPase, isolated from *Acetabularia*, reconstituted into liposomes and tested for a Cl^--translocating activity. A significant increase in Cl^- efflux from the negative and neutral liposomes was observed by addition of ATP in the presence of valinomycin after incorporation of the enzyme by short-term dialysis. The ATP-driven Cl^- efflux was inhibited by the addition of azide, an inhibitor of the ATPase. When chloride was replaced by sulfate, no ATP-dependent sulfate efflux was detectable from the proteoliposomes. Proton-translocating activity of the enzyme was also tested and was found to be negative. Moritani et al.[134] provided evidence from *Acetabularia cliftoni* that the subunit composition was similar to that of F-type ATPases and incorporation of this ATPase into liposomes also provided an ATP-dependent Cl^- transport activity. Collectively, these observations strongly suggested the existence of a Cl^- pump in *Acetabularia*. For the question of the physiological significance of the electrogenic Cl^- pump in *Acetabularia*, Gradmann[80] favors a "Mitchellian" answer. This primary, electrogenic ion pump would create an electrochemical driving force to fuel secondary, electrophoretic (or electroneutral) transport processes, such as uptake of sugars or amino acids. Graves and Gutknecht[82] have provided evidence for an electrogenic Cl^- pump with similar properties in the membrane of *Halicystis*, another marine alga that is related to *Acetabularia*.

One of the most rigorous and definitive proofs for a Cl^- pump's existence and its mode of operation rests with the following group of experiments by Gerencser and his colleagues.[63,79]

V. PHYSIOLOGICAL CHARACTERISTICS OF APLYSIA GUT

A. Tissue

Generally speaking, intestinal preparations of vertebrates bathed in a substrate-free Na^+-containing Ringer solution generate spontaneously transepithelial potential differences of the magnitude 1 to 5 mV, the serosal surface being positive relative to the mucosal surface.[15,27,143] The total active ion absorption in these vertebrate intestines was accounted for by means of active Na^+ absorption in the absence of, or in excess of, active Cl^- absorption.[15,143]

Aplysia californica foregut (crop) bathed in a Na^+-containing seawater medium elicits a spontaneous transepithelial potential difference such that the serosal surface is negative relative to the mucosal surface.[53–56] The total active ion absorption across *Aplysia californica* gut was accounted for by active absorptive mechanisms for both Na^+ and Cl^-, the net absorptive Cl^--transport exceeding the net absorptive Na^+ transport. These results were qualitatively the same as those observed with *Aplysia juliana* gut,[70,71] and account for the negative serosal transepithelial potential difference observed in this gut preparation.

However, past observations suggested that Cl^- absorption was independent of Na^+ absorption.[58,59] Therefore, Cl^- absorption would be independent of Na^+-K^+-dependent ATPase activity. The transepithelial potential difference measured in a Na^+-free seawater medium (Tris Cl) was stable for 3 to 5 h and the electrical orientation of the transepithelial potential difference was serosa negative relative to the mucosal solution. In the absence of Na^+ in the bathing medium, the total active ionic flux was identical to the net mucosal to serosal Cl^- flux.[59] Additionally, mucosally applied thiocyanate or acetazolamide inhibited the transepithelial potential difference and the active absorptive flux of Cl^-. In the absence of an electrochemical potential gradient for Cl^- across the tissue, these observations suggested that there was an active transport mechanism for Cl^-. However, these observations did not delineate location or type of mechanism for the Cl^- active transport.

B. Cellular

Reports of intracellular Cl^- activity (a^i_{Cl}) in vertebrate enterocytes demonstrated that Cl^- was accumulated across the mucosal membrane such that the a^i_{Cl} was two to three times that predicted for electrochemical equilibrium across that membrane.[8,10] These studies concluded that uphill Cl^- movement across the mucosal membrane was coupled to the simultaneous downhill movement of Na^+, and it was this extracellular to intracellular Na^+ electrochemical gradient across the mucosal membrane that was the driving force responsible for intracellular Cl^- accumulation.

The mean a^i_{Cl} determined in *Aplysia californica* foregut epithelial cells bathed in a NaCl seawater medium devoid of substrate was significantly less than that predicted by the electrochemical equilibrium for Cl^- across the mucosal membrane.[56,75] In the absence of Na^+ in the extracellular bathing solution, the mean a^i_{Cl} was also less than that predicted for electrochemical equilibrium for Cl^- across the mucosal membrane.[56,60] So one need not postulate an active transport mechanism for Cl^- in the apical or mucosal membrane of the *Aplysia* foregut absorptive cell because Cl^- transport across this membrane could be driven by the downhill, mucosal to cytosol electrochemical potential gradient for Cl^-. However, once the Cl^- was in the cytosol, it faced a very steep electrochemical potential gradient in its transit across the BLM into the serosal solution.[56,62] Therefore, thermodynamically, the active transport mechanism for Cl^- exhibited in the tissue studies[53,54,59] had to exist in the BLM of the *Aplysia* foregut absorptive cell.

VI. BIOCHEMISTRY OF THE Cl^- PUMP

A. ATPase Activity

Gerencser and Lee[72,74] presented evidence that indicated that the BLM, and only the BLM, of *Aplysia* foregut absorptive cells contains true Cl^--ATPase activity. Biochemical properties of the *Aplysia* foregut absorptive cells BLM-localized Cl^--stimulated ATPase include the following: (1) pH optimum = 7.8; (2) ATP being the most effective nucleotide hydrolyzed; (3) also stimulated by HCO_3^-, SO_3^{-2}, and $S_2O_3^{-2}$, but inhibited by NO_2^-, and no effect elicited by NO_3^- and SO_4^{-2}, (4) apparent K_m for Cl^- is 10.3 m*M* whereas the apparent K_m for ATP is 2.6 m*M*; and (5) an absolute requirement for Mg^{2+} that has an optimal concentration of 3 m*M*.[74] Coincidentally, Cl^- has an intra-

cellular activity[56] in the *Aplysia* foregut epithelial cell approximating its apparent K_m for the Mg^{2+}-dependent Cl^--ATPase, which supports the interrelationship of its physiological and biochemical activities.

Additionally, the ATPase activity stimulated by Cl^- was strongly inhibited by thiocyanate, vanadate, and acetazolamide but not inhibited by ouabain. These results with inhibitors strongly suggested a possible participation by the Cl^--stimulated ATPase in net chloride absorption by the *Aplysia* gut.[74] The finding that anion-stimulated ATPase is inhibited by thiocyanate, but not by ouabain has also been demonstrated in many tissues known to perform active anion transport and to contain anion-stimulated ATPase activity.[108] This inverse parallelism between ouabain insensitivity and thiocyanate sensitivity to Cl^--stimulated ATPase activity and net active Cl^- absorption in the *Aplysia* gut warranted conjecture that the active Cl^- absorptive mechanism could be driven by a Cl^--stimulated ATPase found in the BLM of the foregut absorptive cell. Additional support for this contention rested with the finding that Cl^--stimulated ATPase activity of the BLM was inhibited by vanadate. Vanadate has been shown to inhibit ATPases, which form high-energy phosphorylated intermediates while having no effect on the mitochondrial anion-sensitive ATPase.[3] These results strongly suggested that the Cl^--stimulated ATPase is an ion-transporting ATPase of the "P" variety rather than the "F" or "V" types.

Acetazolamide inhibited Cl^--stimulated ATPase activity in the *Aplysia* gut.[67,74] This finding has also been demonstrated in blue crab gill HCO_3^--ATPase.[125] Although acetazolamide has been shown to be a specific inhibitor of carbonic anhydrase,[129] it has also been demonstrated to be a Cl^- transport inhibitor.[182] Additionally, it had been shown by Gerencser[67] that carbonic anhydrase does not exist in the BLM of the *Aplysia* gut absorptive cell. Thus, the data further strengthen the notion that the Cl^--stimulated ATPase, which is inhibited by acetazolamide, presumably through direct competition, may be involved in net Cl^- transport across the molluscan gut.

B. Transport Activity

Furthermore, Gerencser and Lee[73] demonstrated an ATP-dependent Cl^- uptake in *Aplysia* inside-out gut absorptive cell BLM vesicles that was inhibitable by thiocyanate, vanadate, and acetazolamide. The ATP-driven Cl^- uptake was obtained in the absence of Na^+, K^+, HCO_3^-, or a pH gradient between the intra- and extravesicular space, which is strong suggestive evidence that the Na^+-K^+-ATPase enzyme, Na^+/Cl^- symport, K^+/Cl^- symport, $Na^+/K^+/Cl^-$ symport, Cl^-/HCO_3^-, or Cl^-/OH^- antiport and K^+/H^+ antiport were not mechanisms that are involved in the accumulation of Cl^- within the vesicles.

To further elucidate the electrogenic nature of the ATP-dependent Cl^- transport process, several experimental maneuvers were performed by Gerencser,[62] as follows. First, an inwardly directed valinomycin-induced K^+ diffusion potential, making the BLM inside-out vesicle interior electrically positive, enhanced ATP-driven Cl^- uptake compared with vesicles lacking the ionophore. Second, an inwardly directed FCCP-induced H^+ electrodiffusion potential, making the BLM inside-out vesicle interior less negative, increased ATP-dependent Cl^- uptake compared with control. Third, ATP increased intravesicular negativity measured by lipophilic cation distribution across the vesicular membrane (Table 4). Both ATP and Cl^- appeared to be necessary for generating the negative intravesicular membrane potential, because substituting a non-

TABLE 4
Effect of ATP on Transport Parameters in Basolateral Membrane Vesicles

Experimental condition	Cl^- uptake, nmol/mg protein	n	Vesicular membrane potential difference, mV	n
+ATP	102.7 ± 7.9	3	–34.9 ± 2.5 (12)	12
–ATP	49.7 ± 5.9	3	0.0 ± 5.2 (12)	12
+Nonhydrolyzable ATP analogue (5′-adenylylimidodiphosphate)	59.6 ± 8.3	3	–1.3 ± 0.9 (12)	12
NO_3^- for Cl^- (mole for mole)			+3.0 ± 4.6 (3)	3

Note: Values are means ± SE; n, number of experiments. Reprinted with permission from Gerencser et al.[76]

hydrolyzable ATP analog for ATP, in the presence of Cl^- in the extravesicular medium, did not generate a potential above that of control. Likewise, substituting NO_3^- for Cl^- in the extra- and intravesicular media, in the presence of extravesicular ATP, caused no change in potential difference above that of control. These results also suggested that hydrolysis of ATP is necessary for the accumulation of Cl^- in the vesicles. Furthermore, vanadate, acetazolamide, and thiocyanate inhibited the (ATP+Cl^-)-dependent intravesicular negativity;[76] and in addition, it had been demonstrated that the pH optimum of the Cl^--stimulated ATPase[74] coincided exactly with the pH optimum of 7.8 of the ATP-dependent Cl^- transport in the *Aplysia* foregut absorptive cell BLM vesicles.[62] Therefore, both aspects of the BLM-localized Cl^- pump (ATPase and ATP-dependent Cl^- transport) have the same pH optimum, which suggests that these properties are part of the same molecular mechanism.

C. Sulfhydryl Ligands of Cl^- Pump

It appears that the catalytic, Cl^--stimulated ATPase activity, and its corollary transport components, ATP-dependent Cl^- transport and ATP-dependent membrane potential change in the BLM of *Aplysia* foregut absorptive cells are dependent on intact sulfhydryl ligands.[64,65] P-chloromercuribenzenesulfonate (PCMBS) forms a mercaptide complex with sulfhydryl ligands of the Cl^- pump that inhibit Cl^--stimulated ATPase activity,[64] ATP-dependent Cl^- accumulation, and ATP-dependent membrane potential change in BLM vesicles.[65] These catalytic and transport inhibitions of Cl^- pump activity are totally reversed by dithiothreitol, which is a specific thiol-reducing agent.[146] This result provides strong evidence that the ligands involved in both hydrolysis of ATP and accumulative Cl^- transport are sulfhydryl and not carboxyl, phosphoryl, tyrosyl, or amino.[146] In addition, it appears that the sulfhydryl ligands of the Cl^- pump that are responsible for its catalytic and transport activities are located on the cytoplasmic surface of the BLM of *Aplysia* gut absorptive cells, for PCMBS has been shown to have a very low lipid solubility,[146] and this restricts its action to surface and not intramembranous sulfhydryl ligands.

VII. RECONSTITUTION OF THE Cl^- PUMP

Reconstitution of a membrane protein into a liposome provides one of the few methods needed to rigorously demonstrate the existence of a separate and distinct biochemical and physiological molecular entity. This method also provides evidence that all components of the solubilized protein have been extracted intact. With this premise in mind, Gerencser[66] reconstituted both aspects of the Cl^- pump, that is, the catalytic (ATPase) and transport components from the BLM of *Aplysia* gut absorptive cells. Cl^--stimulated ATPase activity existed significantly above Mg^{2+}-stimulated ATPase activity found in the proteoliposome population extracted and generated with digitonin. Vanadate inhibited this Cl^--stimulated ATPase activity by 99%. From this digitonin-generated proteoliposome population, there is a significant ATP-dependent Cl^- uptake into these proteoliposomes above that of control, and this ATP-dependent Cl^- uptake is also inhibited by vanadate. Not detected in the proteoliposomes solubilized

and formed by digitonin were Na^+/K^+-ATPase, alkaline phosphatase, (Ca^{2+} + Mg^{2+})-ATPase, or cytochrome c oxidase activities and, coupled with a previous observation that FCCP (a protonophore) had a stimulatory and not an inhibitory effect on ATP-driven Cl^- accumulation in the BLM vesicles, suggested that none of these enzymes or eukaryotic vacuolar H^+-ATPases could express Cl^- pump activity. These data also suggested that these two major observations are manifestations of one molecular mechanism: the Cl^- pump. Support of this contention rested with the findings that vanadate (an inhibitor of P-type ATPases) inhibited both Cl^--stimulated ATPase activity and ATP-dependent Cl^- transport in the digitonin-based proteoliposomes. Even though Krogh[118] first coined the term "Cl^- Pump" in 1937, it was not until the reconstitution of all of its components into an artificial liposomal system through the study mentioned above[66] that the existence of this mechanism was proven. Similar reconstitutions of Cl^- pump activity have since been reported in bacteria,[186] alga,[97] and rat brain.[99,185] However, the alga studies[97] are somewhat ambiguous because Cl^- inhibited the Mg^{2+}-ATPase activity despite there being an ATP-dependent Cl^- uptake into the proteoliposome.

A. Molecular Weight

Utilizing polyacrylamide gel electrophoresis (PAGE) techniques to digitonin-generated proteoliposomes containing the Cl^- pump protein from *Aplysia* gut absorptive cells as shown previously,[66] the approximate molecular weight of the Cl^- pump was ascertained.[77] Because both aspects of the Cl^- pump were inhibited by vanadate, it was surmised that the approximate molecular weight of the Cl^- pump of *Aplysia* should be around 100 kDa because vanadate inhibited only P-type ATPases and not F- or V-type ATPases.[139] The alpha-subunit of all P-type ATPases approximates 100 kDa in molecular weight. One major protein band was eluted through PAGE, and its molecular weight was found to be 110 kDa.[77] Recently, similar molecular weights have been obtained for Cl^- pump catalytic units in alga[97,134] and rat brain,[99] confirming the possible E_1-E_2 nature of the ATPase.

B. Reaction Mechanism

The semi-purified protein (Cl^- pump) had been subjected to phosphorylation within the proteoliposome and the reaction sequence and kinetics of the reaction sequence of the enzyme have been determined: Mg^{2+} causing phosphorylation, Cl^- causing dephosphorylation, and all in a time frame consistent with an aspartyl phosphate linkage.[77,78] Hydroxylamine and high pH destablize this phosphorylation, confirming an acyl phosphate bond as an intermediate in the reaction sequence. Vanadate almost completely inhibited the Mg^{2+}-driven phosphorylation reaction, which corroborates the protein catalytic subunit molecular weight of 110 kDa and it also defines the protein as a "P" type ATPase, because vanadate is a transition state inhibitor of phosphate.[139] Figure 1 is an operational model of the reaction sequence of the Cl^- pump.

C. Stoichiometry

The stoichiometry of ATP hydrolyzed to Cl^- transported during a single cycle of the reaction sequence was ascertained

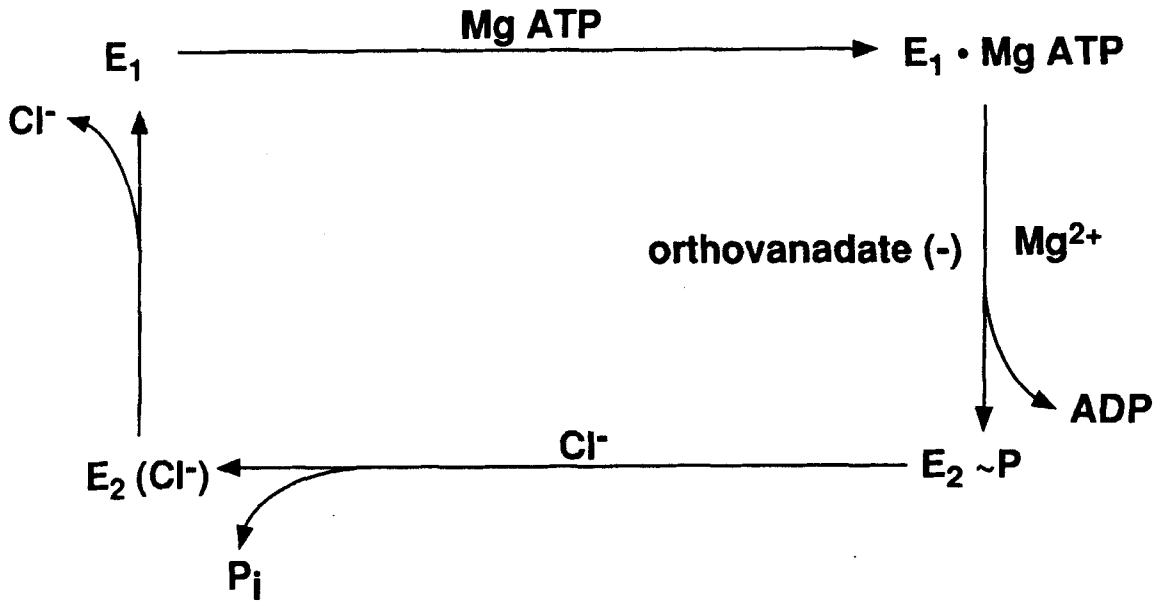

FIGURE 1. Working model of reaction sequence for Cl^- pump. E_1 and E_2 are assumed to be different conformational states of the enzyme because it has been demonstrated that all P-type ATPases have at least two major conformational states.[153,163] (–) represents inhibition by orthovanadate of the Mg^{2+}-driven phosphorylation reaction.

through thermodynamic means.[69] Intracellular concentrations of ATP, ADP, and inorganic phosphate were determined and, coupled with an estimate of the standard free energy of hydrolysis for ATP, the operant free energy for ATP hydrolysis was calculated. Because the operating free energy of the Cl^- pump (electromotive force) was approximately one-half the energy (140 mV) obtained from the total free energy of ATP hydrolysis (270 mV), the only possible integral stoichiometries were one or, at the most, two Cl^- transported per cycle per ATP hydrolyzed. Physiologically, the electrogenic Cl^- pump[62] most likely transports one Cl^- per ATP hydrolyzed per reaction cycle. This increased electrochemical driving force created by the electrogenic nature of the pump could fuel secondary, electrophoretic (or electroneutral) transport processes such as the nutritional uptake of sugars and/or amino acids.[76]

D. Phosphorylation

When the relatively pure proteoliposomal preparation of Cl^--stimulated ATPase[77] was incubated with [γ-^{32}P]ATP, there was a fairly rapid formation of phosphoenzyme that remained stable for 4 min.[68] This finding and the finding that there is a curvilinear relationship between labeled ATP concentrations and phosphoprotein levels suggest that at low ATP concentra-

tions, phosphoprotein formation is directly proportional to the ATP concentration; that is, the system follows Michaelis-Menten kinetics. The relatively high K_m of ATP for the protein (1.25 m*M*) suggests a relatively low affinity of the nucleotide for the enzyme. These findings are similar to what has been found for ATP affinity for fungal and plant P-type proton pumps.[163,176]

The finding of a millimolar K_m value was very surprising in view of well-established micromolar K_m values for ATP reactivity in P-type ATPases from various animal cells. During hydrolysis by brain or electroplax Na^+/K^+-ATPase, ATP reactivity to the enzyme exhibited a K_m below 1 μ*M*.[141] Phosphorylation studies on the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum have yielded ATP-K_m values of approximately 10 μ*M*.[46] The H^+/K^+-ATPase from hog gastric mucosa displays two kinetic components for ATP hydrolysis — one with a K_m near 2 μ*M*, and the other with a K_m near 50 μ*M*.[179]

Despite some exaggeration due to methodologic differences, it seems likely that the disparity of K_m values between the *Aplysia* Cl^--ATPase, fungal and plant proton ATPases, and other cation P-type ATPases is real. One possible interpretation is that the high ATP-K_m ATPases might reflect obligatory activation by nucleotide binding at a nonhydrolyzing, low-affinity binding site, thereby masking the higher-affinity ATP at the catalytic site, such as has been proposed for the Ca^{2+}-ATPase of sarcoplasmic reticulum.[100] This suggestion is circumstantially supported by a slight sigmoidicity in plots of membrane potential, H^+-ATPase activity, and vesicular H^+ transport, against ATP concentration in *Neurospora*.[140,162]

It was also found that adding large amounts of unlabeled ATP concentrations to the incubation medium increased the amount of enzyme dephosphorylation, which suggests that there is at least one binding site for phosphate on the protein. This premise was strengthened by the observation that the ATP molecule also needed to be hydrolyzed in order for this phenomenon to occur. These experiments also suggested that one of the rate-limiting factors for Cl^--ATPase phosphorylation-dephosphorylation is ambient ATP concentrations.[176]

In summation of these phosphorylation experiments, it appears that the Cl^--stimulated ATPase is a P-type ATPase similar in characteristics and reaction scheme to those described for various cation-pumping P-type ATPases present in plasma membranes from a diversity of biological organisms.[139,163] However, it appears that there are two types of this kind of P-type ATPase. One has a high (micromolar) affinity for ATP and is prevalent, for the most part, in plasma membranes of higher animals, whereas the other group has a (millimolar) low affinity for ATP and is present in bacteria,[163] fungi,[162] plants,[176] and molluscs.[68,77]

E. Kinetics

Utilizing a semipurified BLM vesicle preparation containing Cl^--ATPase from *Aplysia* gut, it was demonstrated that ATP, and its subsequent hydrolysis, stimulated both intravesicular Cl^- accumulation and intravesicular negativity with almost identical kinetics.[79] Additionally, in the proteoliposomal preparation the apparent K_m's for ATP-dependent Cl^- uptake, ATP-dependent membrane potential change, and Cl^--stimulated ATPase activity had almost identical Cl^- concentration values to each other (unpublished observations). These values were similar to what had been reported for Cl^--ATPase activity in the *Aplysia* BLM preparation[74] and in rat brain motoneurons.[98]

Similarly, the apparent K_m's of ATP for ATP-dependent Cl^- uptake, ATP-dependent

membrane potential change, and Cl^--stimulated ATPase in the proteoliposomal preparation were similar to each other (unpublished observations) and to the apparent K_m for ATP found for Cl^--ATPase in the BLM of *Aplysia*[74] and for ATP-induced phosphorylation of Cl^--ATPase in the same proteoliposomal preparation of *Aplysia*.[68,77] These kinetic experiments demonstrate the correspondence between overall ATPase activity, Cl^--ATPase phosphorylation, ATP-dependent Cl^- transport, ATP-dependent membrane potential change, and Cl^--ATPase activity which are similar to those characteristics detected in cation-activated and cation-motive ATPases.[139,163]

These kinetics are uniquely significant not only because they are the first and only results obtained with an isolated discreet anion transporter ATPase, but also because they demonstrate the interrelationship, interchangability, and universality between both transport and catalysis of the Cl^- pump.

VIII. CONCLUSIONS

In summary, it is quite apparent that in the past few years there has been an increasing number of convincing studies in a variety of animal tissues that have provided indirect, correlative evidence that active Cl^- transport is primary by nature. The active translocation of Cl^- by an enzyme that directly utilizes the energy from ATP hydrolysis is not unlike that observed in plants.[90,97] Indeed, the evidence for primary active Cl^- transport in these simple living things is almost as convincing as that presented for $(Na^+ + K^+)$-stimulated ATPase and $(Ca^{2+} + Mg^{2+})$-stimulated ATPase in their respective roles for actively transferring Na^+, K^+, and Ca^{2+} across animal plasma membranes. As alluded to by DePont and Bonting,[32] future experimental steps in assuring that an animal Cl^--stimulated ATPase is involved in primary Cl^- transmembrane movement should approximate the following: (1) a specific inhibitor for the enzyme should be found or synthesized (e.g., an antibody), and this inhibitor should be shown to inhibit the transport process; and (2) the Cl^--stimulated ATPase should be biochemically isolated or purified and after its incorporation in liposomes should then be shown to support active Cl^- transport. These demonstrations of reconstitution and phosphorylation of Cl^--ATPase has been shown in the present review, which provides the first direct evidence for the existence of a new P-type ATPase: the Cl^- pump. Future studies should include constructing cDNA probes from a partially sequenced Cl^--ATPase protein that can then transcribe on RNAs of the Cl^- pump protein. The mRNAs should then be shown to translate into Cl^- pump proteins in a non-Cl^- pump containing plasma membrane system.

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